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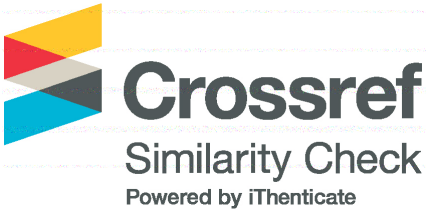

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India	IN	4	2.2
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EDIBLE WILD PLANTS GROWING IN ADJACENT SPONTANEOUS VEGETATION OF ENERGY PLANTATIONS IN SOUTHWEST SLOVAKIA

Lýdia Končeková, Daniela Halmová, Alexander Fehér

ABSTRACT
This paper evaluates the potential and perspectives of wild plant species and macrofungi from short rotation coppice. The research was conducted during the years 2014 – 2018 in stands of short rotation coppice willow and miscanthus grass in southwest of Slovakia. Evaluated wild plant species and macrofungi were divided into four groups (green vegetables, fruits and seeds, flowers and nectar, subterranean parts). The results showed that ground flora of short rotation coppice consisted of 74 edible species from 34 botanical families. *Asteraceae, Rosaceae*, *Poaceae*, *Polygonaceae* and *Cichoriaceae* families were represented the most. From the evaluated categories the most species belonged to the category with consumable aerial parts like leaves and shoots (59 species). The similar representation of species was found in the category of wild fruits and seeds consumed in the raw or preserved state and in category of edible subterranean parts (27 species and 22 species respectively). Principal component analysis showed that the edible parts with the strongest effect on the functional group differentiation were the fruits, seeds and subterranean parts.

Keywords: edible plant; miscanthus; short rotation coppice; SW Slovakia; wild plant

INTRODUCTION
The wild flora has played an essential role in human feeding **(Torija-Isasa and Matallana-Gonzáles, 2016)**. The interest in wild edible plants is not only in terms of increasing dietary balance (sufficient trace elements, vitamins and minerals) but also due to their link to human health **(Tardío, Pardo-de-Santayana and Morales, 2006)**. At present, wild plants play an equally important role in protecting biodiversity and providing various ecosystem services. **Rowe, Street and Taylor (2009)** state that miscanthus and short rotation coppice (SRC) stands have a positive potential impact on biodiversity. Compared to arable land use, they create different structural and functional biotope types with a greater diversity of species due to their longer rotation period, less number of disturbances and chemical inputs and richer spatial structure **(Fry and Slater, 2009; Dauber, Jones and Stout, 2010; Rowe et al., 2011; Verheyen et al., 2014)**.

The benefits that SRC stands can provide consist of provisioning services (production of food, category nutrition – food, crops, wild foods) **(MEA, 2005)**. In the past, wild plant species were collected and used for food, medicine and social issues (during times of famine or conflicts). Nowadays, the increasing interest is based on efforts to provide food security in times of agricultural crisis or use in regional/local cuisine **(Turner et al., 2011; Łuczaj, 2012; Simkova and Polesny, 2015)**. The gathering of wild plants is not only an active living custom **(Christanell et al., 2010)** but it is also a source of cultural identity (cultural services) that is forming an important knowledge about the environment and sustainable living known as traditional ecological knowledge **(Turner et al., 2011)**. While the issues/reviews of the traditional use of edible plants have been evaluated in several works in Slovakia **(Łuczaj, 2012; Stoličná, 2016)** and abroad **(Dogan et al., 2004; Dénes et al., 2012; Di Novella et al., 2013; Guarrera and Savo, 2016; Kuklina and Vinogradova, 2018)**, the prospective use of such species from energy plantations has not yet been studied.

Scientific hypothesis
Taking into account the specific ecological environmental and cultivation-technological conditions of the stands of energy plants, we assumed a high diversity of vascular spontaneous plant species, providing the possibility of occurrence of species with edible parts.

MATERIAL AND METHODOLOGY
The research was carried out on permanent experimental plots established in the agricultural land on a research base of the Slovak University of Agriculture in Nitra in the catastral area of the Kolíňany village (Nitra district area, SW Slovakia). The area belongs to the moderately warm and moderately humid climate region with a sum of

temperatures of 2200 – 2500 °C and an average annual rainfall of 550 – 700 mm. The soil is medium-heavy, the soil type is gley fluvisol. The stands of the species used for energy purposes were established in 2009, consisting of the Swedish willow varieties Tordis (*Salix schwerinii* × *S. viminalis*), Inger (*Salix triandra* × *S. viminalis*) and energy grass (*Miscanthus* ×*giganteus*).

The study of herbaceous species and macrofungi in SRC undergrowth was carried out in the growing periods of 2014 – 2018 at 14-day intervals. The permanent research plots had an area of 2 m x 12 m. The willow varieties were planted from the cuttings in a double-row spacing configuration resulting in a plant density of 8889 plants per ha. The rhizomes of energy grass were planted in 1 x 1 m spacing on an area of 100 m^2 (10,000 plants per ha). A three-year harvest cycle is applied for the willow varieties and the harvest cycle for *M.* × *giganteus* is one year. Based on soil analysis carried out at the beginning of the research period (2014), the soil pH ranged from 7.22 to 7.30. The average humus content was 2.31% and the average nitrogen content was 1479 $mg.kg^{-1}$. The herbicides were applied only prior to the establishment of the research plots in 2009. The vegetation structure was studied using phytocoenological reléves. The presence of species and their relative abundance were assessed using the modified Braun-Blanquet cover-abundance scale for estimating species quantities **(Braun-Blanquet, 1964; Mueller-Dombois and Ellenberg, 1974)**.

Individual identified species were divided into four categories (VEG, FRU, SUB and FLO). The category green vegetables "VEG" consisted of species whose above-ground parts (leaves and stems) were used raw, cooked or fried. Wild fruits and seeds consumed in the raw or preserved form represented the "FRU" category. Plants with edible subterranean parts (rhizomes, roots and tubers) were included in the "SUB" category and species with flowers whose nectar was consumed raw or flowers were added in larger quantities to meals and beverages were categorized as "FLO". In this paper, the classification of species to individual categories was based on a partially modified methodology used in **Łuczaj (2012)** and **Simkova and Polesny (2015)** and the literature sources listed in the References. The nomenclature of the lower and higher plants has been unified according to **Marhold and Hindák (1998)**.

Statistical analysis

Ordination analysis of the species importance in terms of providing edible parts was conducted by the principal component analysis (PCA) in Canoco for Windows version 4.5 and CanoDraw 4.0 **(Braak and Smilauer, 2002)**.

RESULTS AND DISCUSSION

Of the 92 species found in the undergrowth of the trees and plants grown for energy purposes, 74 were edible species. These species represented 73 vascular plants and 1 fungus (Table 1a and Table 1b). The species belonged to 34 botanical families. The list of the edible species included 9 tree species, 4 shrub species, 32 perennial species, 22 annual species and 7 biennial species. The most common families of the edible species were *Asteraceae* and *Rosaceae* (8 species each), *Poaceae* (7 species), *Polygonaceae* and *Cichoriaceae* (5 species each). The most represented was the category of green vegetables with 59 species. The category of fruits (raw or preserved) included 27 species and 22 species belonged to the category of wild plants with edible underground parts (subterranean parts). The least represented was the category of flowers with 17 species.

According to the ethnobotanical review of wild edible plants of Slovakia **(Łuczaj, 2012)**, the most frequently used wild edible plants in Slovakia included the fruits of *Rubus idaeus, Fragaria* spp., *Rubus* subgenus *Rubus, Vaccinium myrtillus, V. vitis-idaea, Fagus sylvatica, Corylus avellana, Prunus spinosa, Pyrus* spp., *Malus* spp., *Crataegus* spp. and the leaves of *Urtica dioica, Rumex acetosa, Chenopodiaceae* species, *Cardamine amara, Glechoma* spp., *Taraxacum* spp. *and Oxalis acetosella*. This species list is similar to our observations (cf. Recorded species of *Rubus* genus, *Prunus spinosa*, *Carataegus* spp., *Urtica dioica*, *Chenopodiacea* species, *Glechoma* spp. and *Taraxacum* spp.) and we can confirm that similar or identical plant species with high edibility potential have been collected for food by local people in Slovakia. The category of green vegetables consisted of plants whose above-ground parts (leaves and stems) are edible raw or cooked, steamed or fried. The most represented were the families *Asteraceae*, *Poaceae* and *Rosaceae* that had the same number of species (6). The second was the family *Cichoriaceae* with 5 species (Figure 1).

Despite the high number of identified species in the category of fruits and seeds (27 species), the most represented family of *Rosaceae* included only 6 species in this category. Other families consisted of two species (fam. *Brassicaceae*, *Poaceae*, *Polygonaceae* and *Solanaceae*) and/or one species with fruits or seeds edible in the raw or preserved state (Figure 2).

The category of edible subterranean parts (roots, rhizomes and tubers) included mostly species of the *Asteraceae* family (4 species). Other families had a similar number of species as the category of fruits and seeds. The families *Brassicaceae*, *Poaceae*, *Rosaceae* and *Violaceae* had two species each. Other families had only one species within this category (Figure 3).

The category of flowers and their nectar eaten raw or flowers added in larger quantities to dishes and beverages consisted of the *Asteraceae* family with three species and the *Violaceae* family with two species. The other families were represented in lower numbers (Figure 4).

The results of the species assessment based on their proportion to the supply of edible parts for human consumption (directly or processed) showed that different species contributed differently in their supply. Differences were apparent also at higher taxonomic levels, e.g. at the genera level and/or the family level. The indirect linear ordination method of PCA (Figure 5) showed that the taxa differentiation was clearly visible on the biplot, therefore the relation detrending was not necessary. The first two component axes of PCA accounted for 65.0% of explained variance. The clusters of species based on the edible part showed that the strongest effect on the differentiation of functional groups (clusters) had the species in the

categories of fruits, seeds and subterranean parts. Categories of flowers and green vegetables showed less effect. The category of flowers was supported by a small number of species (*Capsella bursa-pastoris, Tripleurospermum perforatum* and *Humulus lupulus*). The category of green vegetables was represented by the largest number of species and therefore became a general criterion and not very useful in the formation of functional plant groups (e.g. *Anagallis arvensis, Stellaria media, Lactuca serriola, Mentha longifolia*, etc.). Groups of species were formed also at various transition gradients.

There was a stronger link between the categories of flowers and subterranean parts, while the link was weaker between the categories of green vegetables and fruits. The species of the *Asteraceae* family were scattered relatively evenly but were centred in the axis areas of the VEG, SUB and FLO categories. A similar situation occurred in the case of *Poaceae* species that traced the distribution of *Asteraceae* species in the VEG and SUB categories. However, grasses were surprisingly lacking in the FRU category (edible grains in spikelets are common for the species of the *Poaceae* family). An exception was *Echinochloa crus-galli*. Some typical synanthropic families (e.g. *Chenopodiaceae* and *Amaranthaceae*) have accumulated in the VEG and FRU categories. Representatives of the *Rosaceae* family (*Cerasus, Crataegus, Padus, Prunus, Rosa* and *Rubus* species) behaved similarly, but representatives of the herbaceous species of this family were found in the transition between the SUB and VEG categories (*Geum urbanum* and *Potentilla anserina*). It is an interesting result confirming that there may be different edibility of organs depending on the species lignification even in the same family. Taxa of the *Cichoriaceae* family were typically represented in the VEG category (genera *Lactuca*, *Lapsana* and *Sonchus*).

Our results are in accordance with the synthesis of knowledge on wild food as an ecosystem service in Europe **(Schulp, Thuiller and Verburg, 2014)**. The same is true for Central-Eastern Europe. In the Czech Republic, the use of 175 vascular plant species (the highest number of taxa belonged to families *Rosaceae*, *Asteraceae* and *Ericaceae*) **(Simkova and Polesny, 2015)**, in the part of the Carpathians and the Carpathian Basin (Hungary and adjacent countries) 236 plant species belonging to 68 families **(Dénes et al., 2012)** and in the Pannonian region of Croatia a total of 44 plant taxa belonging to 25 families (the highest number of taxa belonged to families *Asteraceae*, *Lamiaceae* and *Rosaceae*) were recorded **(Žuna Pfeiffer et al., 2019)**. Considering the high number of edible wild plants in the spontaneous vegetation of SRC the perspective of edible wild plants collection is high in comparison with the avarage number of collected edible wild plants in Central-Eastern part of Europe.

Our research evaluated the potential of edible wild plants only but the potential provisioning ecosystem services are not necessarily collected and used by people **(Rasmussen et al., 2016)**. In spite of that, the high value of ecosystem services from small forest patches in agricultural landscapes **(Decocq et al., 2016)** and values of wild foods in agricultural systems **(Bharucha and Pretty, 2010)** are of high importance.

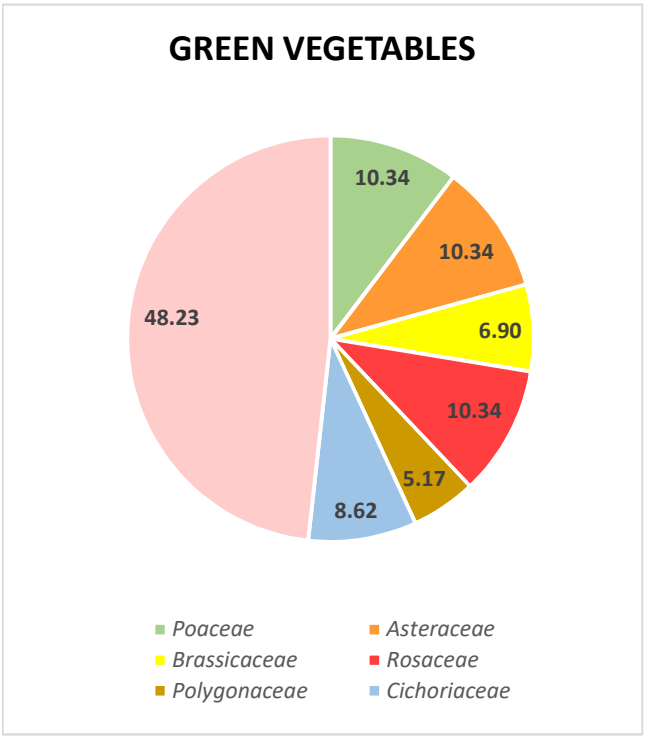

Figure 1 Most representated botanical families in category of green vegetables [in %].

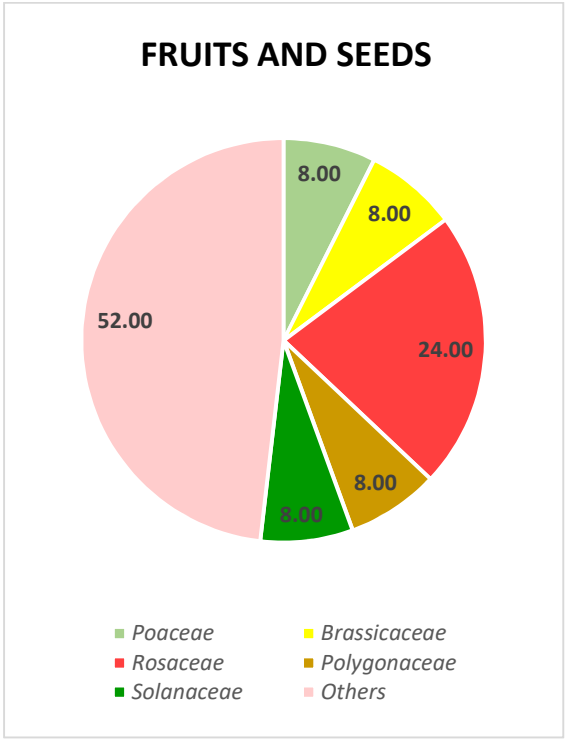

Figure 2 Most representated botanical families in category of fruits and seeds [in %].

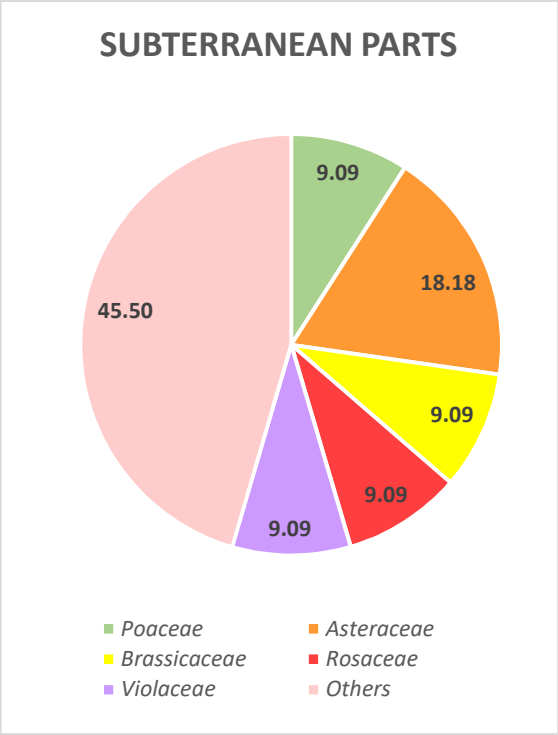

Figure 3 Most representated botanical families in category of subterranean parts [in %].

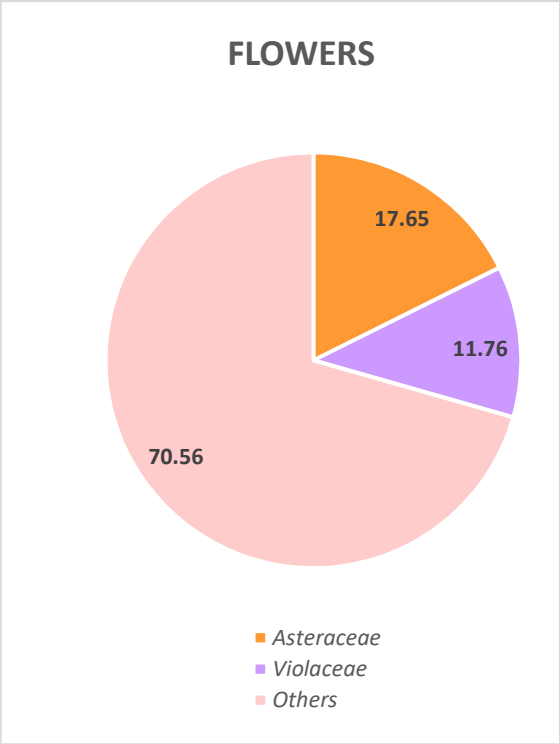

Figure 4 Most representated botanical families in category of flowers [in %].

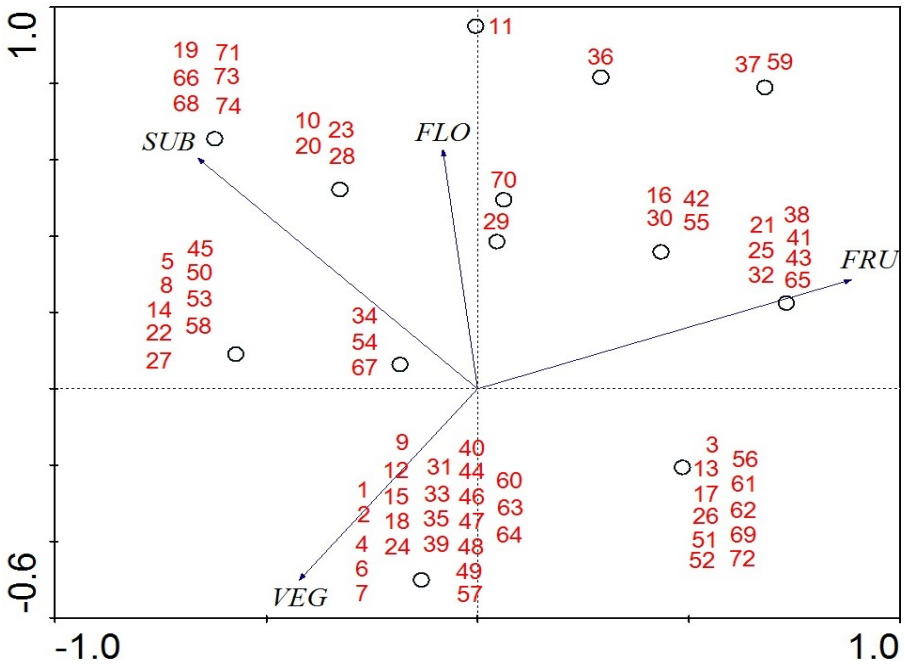

Figure 5 Principal component analysis of functional groups of edible wild plants in energy plantations. The first two axes accounted for 65.0% of explained variance. Note: Ordinal numbers of species are in accordance with Table 1.

Table 1a List of edible wild plants in energy plantations on permanent experimental plots in Kolíňany.

	Species	Family	Use categories
1	*Acer pseudoplatanus*	*Aceraceae*	VEG
2	*Amaranthus powellii*	*Amaranthaceae*	VEG
3	*Amaranthus retroflexus*	*Amaranthaceae*	VEG, FRU
4	*Anagallis arvensis*	*Primulaceae*	VEG
5	*Arctium lappa*	*Asteraceae*	VEG, SUB
6	*Artemisia vulgaris*	*Asteraceae*	VEG
7	*Atriplex patula*	*Chenopodiaceae*	VEG
8	*Bromus sterilis*	*Poaceae*	VEG, SUB
9	*Calamagrostis epigejos*	*Poaceae*	VEG
10	*Calystegia sepium*	*Convolvulaceae*	SUB
11	*Capsella bursa-pastoris*	*Bassicaceae*	VEG, SUB, FLO, FRU
12	*Cardaria draba*	*Bassicaceae*	VEG
13	*Cerasus avium*	*Rosaceae*	VEG, FRU
14	*Cirsium arvense*	*Asteraceae*	VEG, SUB
15	*Clematis vitalba*	*Ranunculaceae*	VEG
16	*Convolvulus arvensis*	*Convolvulaceae*	VEG, FLO, FRU
17	*Crataegus laevigata*	*Rosaceae*	VEG, FRU
18	*Cucubalus baccifer*	*Caryophyllaceae*	VEG
19	*Daucus carota*	*Apiaceae*	VEG, SUB, FLO
20	*Dipsacus fullonum*	*Dipsacaceae*	SUB
21	*Echinochloa cruss-galli*	*Poaceae*	FRU
22	*Elytrigia repens*	*Poaceae*	VEG, SUB
23	*Epilobium hisutum*	*Onagraceae*	SUB
24	*Equisetum arvense*	*Equisetaceae*	VEG
25	*Fallopia convolvulus*	*Polygonaceae*	FRU
26	*Galium aparine*	*Rubiaceae*	VEG, FRU
27	*Geum urbanum*	*Rosaceae*	VEG, SUB
28	*Helianthus annuus*	*Asteraceae*	SUB
29	*Humulus lupulus*	*Cannabaceae*	VEG, SUB, FRU
30	*Hypericum maculatum*	*Hypericaceae*	VEG, FLO, FRU
31	*Chenopodium album*	*Chenopodiaceae*	VEG
32	*Juglans regia*	*Juglandaceae*	FRU
33	*Lactuca serriola*	*Cichoriaceae*	VEG
34	*Lamium purpureum*	*Lamiaceae*	VEG, FLO
35	*Lapsana communis*	*Cichoriaceae*	VEG
36	*Lathyrus tuberosus*	*Fabaceae*	SUB, FRU
37	*Lycium barbarum*	*Solanaceae*	FLO, FRU
38	*Marasmius oreades*	*Tricholomataceae*	FRU
39	*Mentha longifolia*	*Lamiaceae*	VEG
40	*Mercurialis annua*	*Euphorbiaceae*	VEG
41	*Padus serotina*	*Rosaceae*	FRU
42	*Papaver rhoeas*	*Papaveraceae*	VEG, FLO, FRU
43	*Persicaria lapathifolia*	*Polygonaceae*	FRU
44	*Picris hieracioides*	*Cihoriaceae*	VEG
45	*Plantago major*	*Plantaginaceae*	VEG, SUB
46	*Plantago media*	*Plantaginaceae*	VEG
47	*Poa annua*	*Poaceaa*	VEG
48	*Poa pratensis*	*Poaceae*	VEG
49	*Polygonum aviculare*	*Polygonaceae*	VEG
50	*Potentilla anserina*	*Rosaceae*	VEG, SUB
51	*Prunus domestica*	*Rosaceae*	VEG, FRU
52	*Quercus petraea*	*Fagaceae*	VEG, FRU
53	*Raphanus raphanistrum*	*Bassicaceae*	VEG, SUB
54	*Robinia pseudoacacia*	*Fabaceae*	VEG, FLO
55	*Rosa canina*	*Rosaceae*	VEG, FLO, FRU
56	*Rubus caesius*	*Rosaceae*	VEG, FRU

Note: The categories used: VEG – species with edible above-ground parts (leaves and stems), FRU – species with wild fruits and seeds consumed in the raw or preserved form, SUB – plants with edible subterranean parts (rhizomes, roots and tubers), FLO – species with flowers whose nectar was consumed raw or flowers were added to meals and beverages.

Table 1b List of edible wild plants in energy plantations on permanent experimental plots in Kolíňany.

	Species	Family	Use categories
57	*Rumex crispus*	*Polygonaceae*	VEG
58	*Rumex acetosella*	*Polygonaceae*	VEG, SUB
59	*Sambucus nigra*	*Caprifoliaceae*	FLO, FRU
60	*Senecio vulgaris*	*Asteraceae*	VEG
61	*Setaria viridis*	*Poaceae*	VEG, FRU
62	*Solanum nigrum*	*Solanaceae*	VEG, FRU
63	*Sonchus oleracea*	*Cichoriaceae*	VEG
64	*Stellaria media*	*Caryophyllacae*	VEG
65	*Swida sanguinea*	*Cornaceae*	FRU
66	*Symphytum officinale*	*Boraginaceae*	VEG, SUB, FLO
67	*Tanacetum vulgare*	*Asteraceae*	VEG, FLO
68	*Taraxacum* sect. *Ruderalia*	*Cichoriaceae*	VEG, SUB, FLO
69	*Thlaspi arvense*	*Brassicaceae*	VEG, FRU
70	*Tripleurospermum perforatum*	*Asteraceae*	FLO
71	*Tussilago farfara*	*Asteraceae*	VEG, SUB, FLO
72	*Urtica dioica*	*Urticaceae*	VEG, FRU
73	*Viola arvensis*	*Violaceae*	VEG, SUB, FLO
74	*Viola canina*	*Violaceae*	VEG, SUB, FLO

Note: The categories used: VEG – species with edible above-ground parts (leaves and stems), FRU – species with wild fruits and seeds consumed in the raw or preserved form, SUB – plants with edible subterranean parts (rhizomes, roots and tubers), FLO – species with flowers whose nectar was consumed raw or flowers were added to meals and beverages.

CONCLUSION

Based on the results, it can be concluded that:

-SRCs are characterized by a high diversity of species (92 species found), with the vast majority (74 species) of edible species (whole plants or some parts consumable),

-the most numerous were the *Asteraceae*, *Rosaceae* (8) and *Poaceae* (7) families,

the most frequent species (59) were in the VEG category, the above-ground parts of which are edible raw state or processed,

a similar representation of species was found in the FRU (27 species) and SUB (22 species) categories,

the PCA showed that based on the edible part, the most important effect on the differentiation of functional groups had the species in FRU and SUB categories,

a strong correlation was found between the FLO and SUB categories.

The results confirmed the high diversity of vascular plant species (92) with a high proportion of species with edible parts (74).

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THE EFFECT OF PROCESSED TEMPEH GEMBUS TO HIGH SENSITIVITY C-REACTIVE PROTEIN (hsCRP) AND HIGH-DENSITY LIPOPROTEIN (HDL) LEVELS IN WOMEN WITH OBESITY

Desti Ambar Wati, Fika Shafiana Nadia, Muflihah Isnawati, Mohammad Sulchan, Diana Nur Afifah

ABSTRACT
Obesity causes chronic inflammatory reaction is characterized by elevated levels of high sensitivity c-reactive protein (hscrp). Hscrp and hdl could be used as an early marker of cardiovascular disease risk. *Tempeh gembus* contain fiber, unsaturated fatty acids and antioxidants, which can reduce the inflammatory reaction. This study determines the effect of processed *Tempeh gembus* on hsCRP and HDL in obese women. This study included in experimental studies with randomized post-test only control group design involving 40 obese women aged 20 – 50 years. Subjects were randomized into two groups: a control group was given a standard diet low in calories 30 calories/kg body weight, and the treatment group was given a standard diet low in calories 30 calories/kg body weight and *Tempeh gembus* for 28 days. hsCRP and HDL levels were measured before and after the intervention, food intake was measured by using a 3 x 24-hour recall and physical activity (IPAQ form). HsCRP levels were measured using the ELISA method, whereas HDL levels were measured using the CHOD-PAP method. Wilcoxon test (hsCRP levels) and paired *t*-test (HDL levels) used to test differeces before and after intervention each group. Mann Whitney test (hsCRP levels) and independent sample test (HDL levels) used to test differeces before and after intervention between groups. There are differences in hsCRP levels before and after the intervention in the control group ($p = 0.00$) and the treatment group ($p = 0.00$). There are differences in HDL levels before and after the intervention in the control group ($p = 0.00$) and the treatment group ($p = 0.00$). There are differences in the decrease hsCRP levels between the two groups ($p = 0.00$). There are differences in the increase in HDL levels between the two groups ($p = 0.03$). *Tempeh gembus* 150 grams/day can decrease hsCRP levels and increase HDL levels in women with obesity.

Keywords: *Tempeh gembus*; hsCRP; HDL; women; obesity

INTRODUCTION
Obesity is excessive fat accumulation due to an imbalance between energy intake and energy released by the body so that it can interfere with health **(WHO, 2016)**. Obesity is the cause of half the cases of hypertension that increases the risk of cardiovascular disease **(Sizer and Whitney, 2017)**. Cardiovascular disease is caused by narrowing, blockage of coronary arteries and reduced elasticity of blood vessels due to atherosclerosis **(Herrington et al., 2016)**. Atherosclerosis is a progressive disease, even estimated to have occurred since the age of 10 – 20 years with the formation of fat streaks walking slowly and continues to grow by 3% per year since past the age of 20 years. Atherosclerosis occurs due to the interaction of various risk factors, including obesity, hypertension, diabetes mellitus, smoking habits, the aging process, atherogenic dyslipidemia, and pro-inflammatory conditions **(Badimon, Robert and Gemma, 2011)**.

Obesity can also lead to the occurrence of the reaction inflammation due to their secretion of cytokines and pro-inflamator **(Harford et al., 2011)**. Inflammatory reactions cause damage to endothelial function resulting in an increase in stroke volume and cardiac output. Excessive adipose tissue causing chronic inflammatory reactions due to cytokine secretion and proinflamator by adipocyte cells are characterized by increased levels of high sensitivity C-reactive protein (hsCRP), Tumor Necrosis Factor (TNF-α), interferon-gamma (IFN γ) and interleukin-6 (IL-6) **(Guillen et al., 2008; Libby, Ridker and Maseri, 2002)**. hsCRP is a biomarker that is sensitive to the occurrence of inflammation in the body and is a strong predictor of the incidence of cardiovascular system disease **(Tully et al., 2015)**. Increased levels of hsCRP in the long term indicates a process of chronic inflammation **(Pravin and Devang, 2011)**.

Increased adipose tissue in obesity is closely related to the consumption habits of foods that are high in fat and

low in fiber. Excessive fat intake will affect adipose tissue especially visceral fat to express responses to various stimuli, one of which is an increase in the release of free fatty acids by adipose tissue which can stimulate increased secretion of very low density lipoprotein (VLDL) in the liver which in turn results in increased triglycerides, low density lipoprotein (LDL), and decreased high density lipoprotein (HDL) **(Wang and Peng, 2011)**. Low HDL levels are not able to prevent the activation of pro-inflammatory mediators in the form of cytokines such as TNF-α, IL-6 and CRP. The results showed that subjects with HDL levels greater than 60 mg/dL had a lower risk of developing coronary heart disease because an increase of 1 mg/dL HDL levels could reduce the risk of coronary heart disease by 2% in men and 3% in women **(Rajagopal, Suresh and Alok, 2012)**.

Prevention and management of cardiovascular disease can be done by optimizing the consumption of functional foods with high protein fermented foods that are suspected to be able to prevent cardiovascular disease **(Bowen et al., 2018; Anand et al., 2016)**. *Tempeh gembus* is one of Indonesia's original food products made from fermented tofu-based fermentation that functions as a substrate then the tempeh mushroom (*Rhizopus oligosporus)* is added as a microorganism. The main content of *Tempeh gembus* is fiber. The fiber content in 100 grams of *Tempeh gembus* is 3.93 grams, three times more than the fiber content in soybean *Tempeh* **(Sulchan and Endang, 2007; Li, Qiao and Lu, 2012)**. High-fiber diet (≥25 grams of soluble fiber and ≥47 grams of insoluble fiber) per day can reduce the risk by up to 50% of stroke in the population **(Casiglia et al., 2013)**. Other research shows that the *Tempeh gembus* which is processed into snacks, namely *Kerupuk Gembus* contains quite high fiber as much as 54.4 – 67.4% **(Afifah et al., 2019a)**. The fiber content in *tempeh gembus* has anti-inflammatory, anti-carcinogenic effects, can reduce gastrointestinal transit time which is good for treating diarrhea and constipation **(Gropper, Smith, and Groff, 2012)**. Fresh *Tempeh gembus* contains 1.87% fat, 11.09% fiber, 4.90% protein, 89.67% protein digestibility, 14.03% amino acids, 48.07% antioxidant activity, 0.05% genistein, and 0.07% daidzein **(Afifah et al., 2019b)**. In addition *Tempeh gembus* also contains unsaturated fatty acids linoleic acid (21.51%), linolenic aid (1.82%) and oleic (16.72%) **(Sulchan and Endang 2007; Sulchan and Rukmi 2007)**. **Damanik et al. (2018)** showed that the saturated fatty acid content in *Tempeh gembus* (12.55%) was higher than the saturated fatty acid in soybean (12.01%) and tofu residue (12.41%). The content of oleic acid in *Tempeh gembus* can suppress the production of pro-inflammatory cytokines. Giving *Tempeh gembus* in rats fed atherogenic diet can reduce hsCRP levels **(Dewi et al., 2018)**. Research conducted by **Noviana et al. (2018)** on *Tempeh gembus* hydrolyzate which was given 5000 ppm and 8000 ppm bromelin enzymes were able to prevent the microbial activity of *S. aureus*, *B. subtilis*, and *S. mutans*. *Tempeh gembus* also contains fibrinolytic protease-producing microbes, namely *Bacillus pumilus* 2 g (AB968524). Pure fibrinolytic enzymes from Bacillus pumilus 2 g are included in the serine protease group of subtilin which can degrade the α and β chains of fibrinogen quickly so that it has the potential to prevent cardiovascular **(Afifah et al., 2014)**. *Tempeh gembus* has also been shown to reduce oxidative stress. Giving *Tempeh* as much as 25 $g.kg^{-1}$ of Sprague Dawley rats for 28 days on an atherogenic diet, can reduce levels of malonaldehyde and homocysteine. A significant decrease occurred in the administration of fresh *Tempeh gembus* and gembe gembus which were given bromelain enzyme 25 ppm **(Kurniasari et al., 2017)**. Giving *Tempeh gembus* with a dose of 8% and 12% for 5 weeks in experimental animals showed a decrease in levels of total cholesterol, LDL cholesterol and increased HDL **(Afifah et al., 2014)**.

Scientific hypothesis

We investigate several hypotheses in our study:

a. Provision of processed *Tempeh gembus* affects the decrease in levels of high sensitivity c-reactive protein (hsCRP),
b. Provision of processed *Tempeh gembus* has an effect on increasing levels of high density lipoprotein (HDL).

MATERIAL AND METHODOLOGY

This study is a true experimental study using a pre-post randomized control group design **(Sastroasmoro and Ismael, 2011)**. Subjects were divided into two groups, namely the control group and the treatment group. The independent variable of this study was the administration of 150 grams of processed *Tempeh gembus* for 28 days while the dependent variable was the levels of hsCRP and HDL. Researchers have obtained Ethical Clearance from the Ethics Commission of the Faculty of Medicine at Sultan Agung University Semarang, Indonesia with number 33/I/2019/Bioethics Commission.

The study was conducted in March 2019 on 40 female prisoners of Class II Penitentiary in the City of Semarang. Subject retrieval is done based on inclusion criteria, namely women aged 20-50 years, body mass index ≥23 $kg.m^{-2}$, do not have a history or are undergoing liver disease, kidney, cancer, coronary heart disease, stroke, do not smoke, are willing to participate in this study by signing an informed consent .

Physical activity level data were obtained through direct interviews using the IPAQ form and then calculated using the Physical Activity Level (PAL) formula **(WHO, 2011)**. Categorizing the level of physical activity is light (1.40 – 1.69 units), moderate (1.70 – 1.99 units), and heavy (2.00 – 2.40 units). Data on Body Mass Index were obtained based on measurements of body weight and height. Data on nutrient intake was obtained through direct interviews using a food recall form and then analyzed using Nutrisurvey software.

Determination of the subject of this study using consecutive sampling method, and found as many as 73 people were willing to have blood drawn for the initial screening process. There were 40 subjects who met the inclusion criteria which were then divided into 2 groups: one control group and one treatment group with each group consisting of 20 subjects. The control group was given a diet limiting the intake of 30 calories/kg body weight/day while the treatment group as many as 20 people were given a dietary intake limiting the intake of 30 calories/kg body weight/day + processed 150 grams of *Tempeh gembus*/day.

Food intake restriction intervention is done by reducing

Figure 1 Tofu waste fresh.

Figure 2 Preparation of *Tempeh Gembus*.

Figure 3 Addition *Tempeh* yeast.

Figure 4 *Tempeh Gembus* has been ermented for 36 hours.

Figure 5 *Tempeh Gembus* Balado.

Figure 6 *Tempeh Gembus* Oseng.

Figure 7 *Tempeh Gembus* Bacem.

Figure 8 *Tempeh Gembus* Pepes.

Figure 9 *Tempeh Gembus* Satay.

to the nutritional value **(Redman and Ravussin, 2011)**. Limitation of food intake is given in stages as much as 30 kcal.kg^{-1} body weight/day through food menus provided by the correctional institution which refers to a low calorie diet for obesity by considering the gender and physical activity of the subject **(Wahyuningsih, 2013)**. The Japan Atherosclerosis Society (JAS) recommends limiting food intake for obese subjects with mild physical activity levels of 25 – 30 kcal.kg^{-1} body weight day, 30 – 35 kcal.kg^{-1} body weight/day for subjects with moderate physical activity levels and >35 kcal.kg^{-1} body weight/day for subjects with heavy levels of physical activity **(Kinoshita et al., 2017)**. Interventions in the two groups were carried out for 28 days.

The independent variable of this study was the administration of 150 grams of processed *Tempeh gembus*. *Tempeh gembus* which will be given as treatment material is made by researchers and the team, in the Laboratory of Food Technology laboratory polytechnic health of Semarang using tofu waste obtained from tofu craftsmen food portions while still paying attention in the Cinde-Lamper region of Semarang City, *Tempeh* yeast used is Raprima yeast. *Tempeh gembus* is processed into 5 types of cuisine, namely: bacem, satay, oseng, pepes, and balado.

The dependent variable of this study is the levels of hsCRP and HDL. HsCRP levels were measured using the Enzyme Linked Immunosorbent Assay (ELISA) method **(Crowther, 2009)**. Whereas HDL levels were measured by laboratory workers using the Cholesterol Oxidase-Peroxidase Aminoantipyrine Phenol (CHOD-PAP) method **(McPherson and Pincus, 2016)**. Blood samples were taken by Semarang CITO Laboratory officials twice, namely on the first day before being given an intervention and 1 day after the intervention (the 29th day).

Statistic analysis

Data were analyzed using the version 16.0 of Statistical Package for the Social Sciences (SPSS). Differences in hsCRP levels before and after treatment were analyzed using the Wilcoxon test because the data were not

normally distributed. Differences in treatment effect between the two groups were analyzed using the Mann Whitney test. Differences in HDL levels before and after treatment were analyzed using paired *t*-test because the data were normally distributed. Differences in treatment effect between the two groups were analyzed using the independent sample test.

RESULTS AND DISCUSSION

Subject characteristics consisting of age, level of physical activity, Body Mass Index before and after treatment are presented in Table 1. All subjects in the study were in the age group of 21-50 years. The mean age in the control group (35.05 ±8.54 years) was lower than in the treatment group (36.50 ±9.37 years). The Mann Whitney test showed that there was no significant difference in age between groups (p = 0.64), so age was not a confounding variable in the study. The mean level of physical activity during the study in the control group (1.52 ±0.17 units) was higher than the treatment group (1.48 ±0.10 units). The Mann Whitney test showed that there was no difference in the average level of physical activity between groups (p = 0.84), so the level of physical activity was not a confounding variable in the study. Based on **WHO (2011)**, the level of subject activity in this study was included in the mild category (1.40 – 1.69 unit). Table 1 also shows that the average Body Mass Index (BMI) before and after the study in the control group (30.00 ±5.61 $kg.m^{-2}$; 29.63 ±5.42 $kg.m^{-2}$) than the treatment group (28.22 ±2.49 $kg.m^{-2}$; 27.32 ±2.50 $kg.m^{-2}$). The Mann Whitney test showed that there was no difference in mean Body Mass Index before (p = 0.51) and after research (p = 0.16), confounding in the study.

Table 2 shows data on energy, protein, fat and carbohydrate intake patterns before the study. The mean energy intake before the study in the control group (2306.96 ±545.24 kcal) was higher than in the treatment group (2057.70 ±241.06 kcal), the Mann Whitney test showed that there were significant differences in energy intake between groups (p = 0.01) so that energy intake before the study becomes confounding variable. The mean protein intake before the study in the control group (84.86 ±19.08 grams) was higher than the treatment group (72.59 ±19.16 grams), the independent sample test showed that there were significant differences in energy intake between groups (p = 0.04) so that protein intake before it can become confounding variables. The mean fat intake before the study in the control group (86.8 ±31.84 grams) was higher than the treatment group (60.57 ±18.75 grams), the independent sample test showed that there were significant differences in energy intake between groups (p = 0.00) so that fat intake before the study can be a confounding variable. The mean carbohydrate intake in the control group (296.79 ±77.45 gram) was higher than the treatment group (276.42 ±35.50 gram), but the independent sample test showed no difference in carbohydrate intake before the inter-group study (p = 0.29) so that carbohydrate intake before the study did not become a confounding variable.

Nutrient intake data subject control and treatment groups during the study are presented in Table 3. Table 3 Based on average energy intake in the control group (1924.35 ±218.62 kcal) is higher than that of the treatment group (1883.81 ±187.89 kcal), independent sample test showed that there were no significant differences in energy intake between groups (p = 0.53) so that energy intake was not a confounding variable in the study. The mean protein intake in the control group (p = 0.53) was higher than the treatment group (96.21 ±10.93 gram), the independent sample test showed no significant differences in protein intake between groups (p = 0.53) so that protein intake is not a confounding variable in the study. The mean fat intake in the control group (42.76 ±4.85 grams) was higher than the treatment group (41.86 ±4.17 grams), the independent sample test showed no significant difference in fat intake between groups (p = 0.53) so that fat intake does not become a confounding variable in the study. The mean carbohydrate intake in the control group (288.611 ±32.75 grams) was higher than the treatment group (282.54 ±28.16 grams), the independent sample test showed no significant difference in carbohydrate intake between groups (p = 0.53) so that carbohydrate intake is not a confounding variable in the study.

Table 4 shows the levels of hsCRP before and after the intervention. In the control group, the mean hsCRP level before the intervention was 7.31 ±0.75 $mg.L^{-1}$ whereas after the intervention the mean hsCRP level became 5.65 ±0.88 $mg.L^{-1}$. In the treatment group, the mean hsCRP level before intervention was 5.63 ±1.23 $mg.L^{-1}$, whereas after the intervention was 3.69 ±1.35 $mg.L^{-1}$. There was a significant difference between the mean levels of hsCRP before and after the intervention in the two groups (p = 0.00; p = 0.00). The mean hsCRP level before the intervention in the control group (7.31 ±0.75 $mg.L^{-1}$) was higher than the treatment group (5.63 ±1.23 $mg.L^{-1}$), there was a significant difference to the average hsCRP level before the intervention between the two group (p = 0.00). The mean hsCRP level after intervention in the control group (5.65 ±0.88 $mg.L^{-1}$) was higher than the treatment group (3.69 ±1.35 $mg.L^{-1}$), there was a significant difference to the average hsCRP level after the intervention between the two group (p = 0.00). There was a significant difference in the decrease in hsCRP levels after the intervention in both groups (p = 0.03).

In this study, the mean hsCRP level in the treatment group decreased by 1.94 ±0.29 $mg.L^{-1}$. This shows that giving as much as 150 grams of *Tempeh gembus* per day for 28 days is effective in reducing levels of hsCRP. The decrease in hsCRP levels in the treatment group can be caused by the presence of fiber in *Tempeh gembus*. Low fiber intake can increase proinflammatory cytokines IL-6), TNF-α, and IL-18. Increasing IL-6 can consistently increase CRP levels. High fiber intake can reduce fat oxidation resulting in decreased inflammation. Fiber is a protective factor to counter increasing CRP levels **(Ma et al., 2006)**. Other studies have shown that fiber in fermented soybeans can reduce cholesterol levels so that it contributes positively to the anti-inflammatory effect. The fiber in fermented soybean consists of several monosaccharides including glucose, arabinose, galactose, and uronic acid which are components of cellulose and non-cellulose polysaccharides. The main non-cellulose polysaccharide from soybean fiber is arabinogalactant. The positive effect on the anti-inflammatory effect can be seen significantly in decreasing the levels of C-Reactive Protein **(Kim et al., 2014)**.

Besides fiber, *Tempeh gembus* also contains antioxidants in the form of isoflavones (daidzein and genistein) and unsaturated fatty acids (oleic, linoleic and linolenic fatty acids) which include essential fatty acids **(Sulchan and Endang 2007; Sulchan and Rukmi 2007)**. The anti-inflammatory mechanism by isoflavones is carried out by inhibiting the NF-kB transcription system and modulating arachidonic acid (AA) metabolism and Nitric Oxide (NO) production by inhibiting protein levels and the activity of proinflammatory enzymes (phospholipase A2 (PLA2), lipoxygenase (LOX, COX-2, and iNOS) **(Jie et al., 2016)**. This study is in line with other studies that show that interventions with soy-based foods can reduce high sensitivity levels of C-Reactive Protein by 25% **(Kone, 2014)**. Apart from isoflavones, antioxidant activity *Tempeh gembus* probably derived from amino acids/peptides bioactive. *Tempeh gembus* containing amino acids such as tyrosine, methionine, histidine, lysine, cysteine and tryptophan. Activity of antioxidants in soybean gembus with ABTS method was 63.14 ±1.16% **(Agustina et al., 2018)**.

Table 5 shows data on HDL levels before and after the intervention. In the control group, the mean HDL level before the intervention was 29.25 ±5.05 mg.dL^{-1} whereas after the intervention the mean HDL level was 35.45 ±3.79 mg.dL^{-1}. In the treatment group, the mean HDL level before the intervention was 32.50 ±5.62 mg.dL^{-1} whereas after the intervention the average HDL level was 41.90 ±2.73 mg.dL^{-1}. There was a significant difference between the mean HDL levels before and after the intervention in the two groups (p = 0.00; p = 0.00). The mean HDL levels before the intervention in the control group (29.25 ±5.05 mg.dL^{-1}) were lower than the treatment group (32.50 ±5.62 mg.dL^{-1}), there were no significant differences in the mean HDL levels before the intervention between both groups (p = 0.06). The mean HDL levels after the intervention in the control group (35.45 ±3.79 mg.dL^{-1}) were lower than the treatment group (41.90 ±2.73 mg.dL^{-1}), there were significant differences in the mean HDL after the intervention between the two groups (p = 0.00). There was a significant difference in the increase in HDL levels after the intervention in both groups (p = 0.00). In this study, the mean HDL levels in the treatment group increased by 9.40 ±4.48 mg.dL^{-1}. The provision of 150 grams of processed Tempeh gembe for 28 days is effective in increasing HDL levels in the treatment group.

The main content of *Tempeh gembus* is fiber. High fiber intake can increase the excretion of bile acids and cholesterol through feces thereby reducing bile acids to get back into the liver. The reduction of bile acids to the liver causes an increase in the use of cholesterol to bile acids so that it has an effect on increasing HDL **(Buse, Kenneth and Harles, 2017)**. Besides fiber, the increase in HDL levels in the treatment group was due to the presence of flavonoids in *Tempeh gembus.* Flavonoids can increase the amount of Apolipoprotein A-1. Apolipoprotein A-1 acts as an enzyme cofactor for LCAT and as a ligand of interaction with lipoprotein receptors in tissues in HDL. An increase in Apolipoprotein A-1 is expected to increase HDL levels **(Gropper, Smith, and Groff, 2012)**. According to the American Association of Clinical Endocrinologists **(AACE, 2012)** HDL levels of 60 mg.dL^{-1} can reduce the risk of coronary heart disease **(Jellinger et al, 2012)**. However, in this study there were no respondents with HDL levels reaching 60 mg.dL^{-1} after the intervention. Increased levels of HDL that do not reach optimal values are caused by several factors, one of which is exercise **(Whitney and Sharon, 2015)**. Other studies have shown that adult women who exercise regularly, such as aerobic exercise three times a week, can experience increased levels of HDL **(Wang and Peng, 2011)**. WHO states that aerobic exercise performed by adults aged 18-64 years with moderate intensity for 150 minutes/week or high intensity for 75 minutes/week can increase HDL levels and be beneficial for heart health **(WHO, 2011)**. Regular exercise can improve the work function of Apolipoprotein A-1 as an HDL receptor in reducing cholesterol from blood vessel walls **(Kingwell and Michael, 2013)**. In this study, respondents only did aerobics once a week so this might be the cause of HDL cholesterol levels not reaching optimal values. In addition to lack of exercise, respondents' physical activity is also included in the mild category. The types of activities most frequently carried out by respondents were sleeping, watching TV, sweeping, washing clothes, cooking and making batik.

Changes in HDL and hsCRP levels also occurred in the control group. This is presumably due to the provision of restriction of food intake through a low calories diet of 30 calories per kg body weight/day in the control group. Based on the results of the study, it is known that the control group's diet before the intervention was 2306.96 kcal while during the intervention it decreased to 1924.35 kcal.

Changes in calorie intake resulted in an increase in HDL levels of 6.20 ±2.35 mg.dL^{-1}, from 29.25 ±5.05 mg.dL^{-1} to 35.45 ±3.79 mg.dL^{-1}. Foods that have been consumed will undergo metabolic processes and produce energy in the form of adenosine triphosphate (ATP) to carry out physical activity **(Rodwell et al., 2018)**. When doing physical activity, energy needs will increase, so if glucose as the main energy source is insufficient there will be an increase in fat metabolism. This causes a decrease in body fat percentage and an increase in HDL cholesterol **(Whitney and Sharon, 2015)**. Research on obese subjects shows that food intake restrictions affect changes in lipid profile, including increased levels of HDL **(Fothergill et al., 2016)**. Food intake restrictions are also able to protect against age-related diseases including inflammation by reducing oxidative stress **(Omodei and Luigi, 2011; Rebrin, Michael and Rajindal, 2011)**.

Interventions in the form of limiting food intake in the control group also resulted in a decrease in hsCRP levels of 1.65 ±0.57 mg.L^{-1}, from 7.31 ±0.75 mg.L^{-1} to 5.65 ±0.88 mg.L^{-1}. A decrease in subject's hsCRP levels can be caused by a decrease in fat intake. It is known that the mean fat intake in the control group before the intervention is 86.58 ±31.84 grams to 42.76 ±4.85 grams after the intervention. Fat intake is excessive will affect visceral fat to express a response to various stimuli one of them is an increase in spending of free fatty acids by adipose tissue that can stimulate increased secretion of VLDL in the liver which in turn will result in an increase in triglycerides, LDL and decrease HDL **(Gropper, Smith and Groff, 2012; Wang and Peng, 2011)**. This increase

will trigger the release of HDL from the liver to carry cholesterol in the circulation (reverse cholesterol transport).

HDL is esterified into cholesterol esters which can be directly carried to the liver to be directly excreted or exchanged with triglycerides from VLDL and chylomicrons. When cholesterol esters are excessive, triglyceride-rich HDL (low density HDL) is broken down by hepatic lipase, thus reducing circulating HDL levels. As the result the reverse cholesterol transport process is reduced and cholesterol levels in the circulation and tissue increase **(Murray, Granner and Rodwell, 2017)**. In one condition, an increase in excess cholesterol levels in the circulation causes an abnormal reaction that causes the activation of the scavenger macrophage. This macrophage is responsible for cleaning cholesterol and low-density

Tabel 1 Respondent Characteristics.

Respondent Characteristics		Control Group			Treatment Group			
		Mean ±*SD*	Median	Min-Max	Mean ±*SD*	Median	Min – Max	*p*
Age (years)		35.05 ±8.54	34.00	21 – 50	36.50 ±9.37	39.00	21 – 48	0.64*
Body Mass Index ($kg.m^{-2}$)	Before	30.00 ±5.61	28.40	25.10 – 47.67	28.22 ±2.49	27.73	25.08 – 33.23	0.51*
Body Mass Index ($kg.m^{-2}$)	After	29.63 ±5.42	27.99	25.00 – 47.03	27.32 ±2.50	27.14	24.15 – 32.47	0.16*
Level of physical activity (unit)	During	1.52 ±0.17	1.42	1.40 – 1.79	1.48 ±0.10	1.43	1.40 – 1.73	0.84*

Note: * Mann Whitney test.

Table 2 Diet Before Research.

Respondent Characteristics	Control Group			Treatment Group			
	Mean ±*SD*	Median	Min – Max	Mean ±*SD*	Median	Min-Max	*p*
Energy (kcal)	2306.96 ±545.24	2284.65	916.40 – 3328.10	2057.70 ±241.06	1954.68	1823.6 – 2714.50	0.01*
Protein(grams)	84.86 ±19.08	87.90	45.90 – 118.40	72.59 ±19.16	71.65	39.50 – 111.30	0.04**
Fat (grams)	86.58 ±31.84	82.55	26.90 – 131.30	60.57 ±18.75	59.41	30.80 – 109.00	0.00**
Carbohydrate (grams)	296.79 ±77.45	289.90	122.00 – 451.10	276.42 ±35.50	274.33	216.80 – 364.70	0.29**

Note: * Mann Whitney test; ** independent sample test.

Table 3 Nutrition Intake During Research.

Respondent Characteristics	Control Group			Treatment Group			
	Mean ±*SD*	Median	Min – Max	Mean ±*SD*	Median	Min – Max	*p*
Energy (kcal)	1924.35 ±218.62	1955.07	1456.65 – 2457.00	1883.81 ±187.89	1895.40	1512.81 – 2299.05	0.53**
Protein(grams)	96.21 ±10.93	97.75	72.83 – 122.85	94.19 ±9.39	94.77	75.64 – 114.95	0.53**
Fat (grams)	42.76 ±4.85	43.45	32.37 – 54.60	41.8 6 ±4.17	42.12	33.62 – 51.09	0.53**
Carbohydrate (grams)	288.61 ±32.75	293.26	218.50 – 368.55	282.54 ±28.16	284.31	226.92 – 344.86	0.53**
Tempeh Gembus (%)	0.00 ±0.00	0.00	0.00 – 0.00	61.37 ±19.05	56.88	35.88 – 94.90	0.00**

Note: ** independent sample test.

Table 4 hsCRP levels before and after the intervention.

hsCRP levels ($mg.L^{-1}$)	Control Group (n = 20)			Treatment Group (n = 20)			*p*
	Mean ±*SD*	Median	Min – Max	Mean ±*SD*	Median	Min – Max	
Pre Intervention	7.31 ±0.75	7.15	6.20 – 8.90	5.63 ±1.23	5.35	4.40 – 9.30	0.00*
Post Intervention	5.65 ±0.88	5.60	4.40 – 7.30	3.69 ±1.35	3.40	2.40 – 7.70	0.00*
Δ	1.65 ±0.57	1.55	2.90 – 0.20	1.94 ±0.29	2.00	2.40 – 1.00	0.03*
p		0.00***			0.00 ***		

Note : *p*-value <0.05 = significant*;* * Mann Whitney test*;* *** Wilcoxon.

Table 5 HDL levels before and after the intervention.

HDL levels (mg/dL)	Control Group (n = 20)			Treatment Group (n = 20)			*p*
	Mean ±*SD*	Median	Min – Max	Mean ±*SD*	Median	Min – Max	
Pre Intervention	29.25 ±5.05	29.00	20.00 – 38.00	32.50 ±5.62	34.00	21.00 – 39.00	0.06**
Post Intervention	35.45 ±3.79	36.00	27.00 – 42.00	41.90 ±2.73	42.00	36.00 – 47.00	0.00**
Δ	6.20 ±2.35	6.00	2.00 – 10.00	9.40 ±4.48	8.00	2.00 – 20.00	0.00**
P		0.00****			0.00 ****		

Note *p*-value <0.05 = significant*;* ** independent sample test*;* **** paired *t* test.

HDL from circulation by phagocytosis. Macrophages that are full of cholesterol will then become foam cells that cause activation of pro-inflammatory cytokines (IL-1, IL-6, and TNF α). Activation of pro-inflammatory cytokines is an early sign of inflammation. Continued inflammation will cause CRP expenditure from the liver **(Pitsavos et al., 2006)**. Other studies have shown that low fat intake can inhibit cytokine release and reduce levels of hsCRP in the blood **(Camhi et al., 2010)**. This is due to adipose tissue reducing the expenditure of free fatty acids causing a decrease in total cholesterol, LDL, triglyceride levels and an increase in HDL that affect macrophages, thereby impacting on decreasing hsCRP levels **(Murray, Granner and Rodwell, 2017)**.

Intervention in the form of limiting long-term food intake has a very strong protective effect on the risk of atherosclerotic cardiovascular disease (CVD) as evidenced by a decrease in blood pressure, LDL cholesterol, hsCRP, IL-6, TNF-α, and an increase in HDL cholesterol levels **(Dolinsky and Dyck, 2011)**. Restricted food intake has been shown to improve mitochondrial function, reduce oxidative stress and increase nitric oxide production involved in the prevention of atherosclerosis, reduction in blood pressure, weakening of left ventricular hypertrophy, resistance to myocardial ischemic injury and prevention of heart failure **(Weiss and Luigi, 2011)**.

CONCLUSION

The administration of processed *Tempeh gembus* for 28 days can reduce high sensitivity c-reactive protein (hsCRP) by 1.93 $mg.L^{-1}$ and increase HDL levels by 9.40 $mg.dL^{-1}$ in obese women in penitentiary class II Semarang city, Indonesia. Changes in hsCRP and HDL levels also occurred in the control group. This is thought to be due to a standard diet of 30 calories/kg body weight/day for 28 days.

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INTERACTION OF POLYPHENOLS EXTRACT FROM *POLYGONUM MULTIFLORUM* THUNB. ROOTS WITH GELATIN AND TOXICITY OF EXTRACT IN MICE

Le Pham Tan Quoc

ABSTRACT
The roots of *Polygonum multiflorum* Thunb. (Vietnamese name: Ha-thu-o-do, HTOD) are used in processed form or the raw state in traditional Vietnamese medicine for many diseases and in extract form in the food industry. Some studies pointed out that HTOD extract had toxicity in humans. However, the toxicity of this herb plant currently remains unclear. In addition, this material contained a large amount of bioactive compounds, especially phenolic compounds. They have a strong antioxidant capacity and they can also interact with many different substrates such as protein, enzyme, lipid and carbohydrate. In this study, the received extracts from HTOD had the polyphenols concentrations of 415, 277, 208 and 166 (mg GAE.L^{-1}), respectively. Besides, we only evaluated the gelatin-polyphenols interaction and the toxicity of HTOD extract in Swiss mice. The results show a strong gelatin-polyphenols interaction and no acute or subacute toxicity in mice. The polyphenols extract of HTOD at the concentration tested in this study is safe to use in food.

Keywords: Interaction; gelatin; polyphenols; root; toxicity

INTRODUCTION
Polygonum multiflorum Thunb. is a wild herbal plant distributed throughout the mountainous regions of North Vietnam (Cao Bang, Lang Son, Lai Chau, Hoa Binh province, etc.). It is known as Ha-thu-o-do (HTOD) in Vietnamese. In addition, HTOD is found in many other Asian countries such as China, Korea, Japan, etc. Ethnomedical uses of HTOD have been recorded for many centuries, and it contains more than 100 chemical bioactive compounds, for example, tannins, anthraquinones, stilbenes, flavonoids, phospholipids **(Lin et al., 2015)**, saponins, and alkaloids **(Quoc and Muoi, 2018)**. Thus, it can be used as a traditional spice in Chinese food **(Li and Gao, 2015)** or drugs to prevent some diseases, such as certain forms of cancer **(Way et al., 2014)**, and for its anti-aging effects, tonic tension **(Lim et al., 2014)**, and antioxidant activity **(Wang et al., 2008)**. Nowadays, polyphenols extract from HTOD is used in food processing, for example, it serves as an antioxidant during the storage of minced red tilapia **(Le and Nguyen, 2018)** or combine with edible film (alginate) to store fresh-cut papaya **(Quoc and Muoi, 2016)**. Moreover, HTOD has also been used in wine processing **(Hoang and Thuat, 2015)**.

There are many methods to extract phenolic compounds from HTOD with various solvents (water, acetone, methanol, ethanol, etc.), such as the decoction method **(Li et al., 2007)**, microwave-assisted extraction **(Quoc and Muoi, 2015)**, ultrasound-assisted extraction **(Wu et al., 2012)**, pectinase-assisted extraction **(Quoc and Muoi, 2017)**, etc. The total polyphenols content, antioxidant capacity, and type of phenolic compounds in all methods are significantly different. Thus, these results can strongly affect the protein-polyphenols interaction and toxicity of polyphenols extract in mice.

Recently, many studies reported that HTOD can have hepatotoxic effects **(Huang, Zhang and Sun, 2011; Wu et al., 2012)**; other studies noticed that HTOD is good for the liver **(Huang et al., 2007; Bhadauria, 2010)**. The results of the above-described studies appear to be contradictory. Until now, there have been no studies on the interaction of gelatin with polyphenols extract from HTOD. Therefore, the main aim of this research was to investigate the gelatin-polyphenols interaction and toxicity of polyphenols extract in mice.

Scientific hypothesis
The objective of this study was to determine the capacity for interaction between polyphenols extracts of *Polygonum multiflorum* Thunb. roots and protein (gelatin). This interaction results in precipitation of the protein. An additional objective of the study was to determine the effect of the toxicity of various polyphenols extract

concentrations in mice. We are expecting an insignificant effect of the extract on acute and subacute toxicity in mice.

MATERIAL AND METHODOLOGY

Extract preparation

Polygonum multiflorum Thunb. roots were harvested from Cao Bang province (Vietnam). The roots were then cleaned with tap water, sliced, and dried at 60 °C until the moisture level was less than 12%. The slices were then ground into a fine powder (diameter less than 0.5 mm) and vacuum-packed. Polyphenols from the dried powder of *Polygonum multiflorum* Thunb. roots were extracted in a microwave system with an acetone concentration of 57.35%, solid/solvent ratio of 1/39.98 (w/v), extraction time of 289 sec, and microwave power of 127 W. The crude extract was filtered through Whatman paper **(Quoc and Muoi, 2015)**. The filtered extract was evaporated at 45 °C until the solvent was completely removed and the extract was used for the preparation of 415, 277, 208 and 166 mg GAE.L^{-1} solution in distilled water.

Chemicals and reagents

Folin-Ciocalteu reagent and gallic acid were purchased from Merck (Germany). All organic solvents and other chemicals were of analytical reagent grade.

Determination of total polyphenols content (TPC)

The TPC in the extracts was slightly modified and determined by the Folin-Ciocalteu colorimetric method **(Siddiqua et al., 2010)**. The results were based on a standard curve obtained with gallic acid. TPC was expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE.g^{-1} DW) or per gram of solution volume (mg GAE.L^{-1}).

Interaction of polyphenols extract with gelatin

Reactions took place in 10 mL volumetric flasks. Polyphenols were dissolved in water to 415, 277, 208 and 166 (mg GAE.L^{-1}). Gelatin solutions in water, at concentrations varying from 30 to 120 mg.mL^{-1} were prepared. Polyphenols and gelatin solutions were mixed quantitatively in flasks with shaking. After standing for 24 h at 25 °C, the mixture was centrifuged (3000 rpm, 20 min), and the suspended substances (reaction products) were removed. The supernatant was analyzed at 280 nm in a UV spectrophotometer. Assuming that a polyphenols solution with a fixed concentration has an initial absorbance (A_o), and after interaction with gelatin the absorbance decreases to A, RA can be defined as $RA = (A_o-A)/A_o$ **(Bi, He and Haslam, 1995)**.

Animals

Male and female Swiss mice (approximately 22 g) were obtained from the Pasteur Institute (Ho Chi Minh city, Vietnam). All mice were maintained in plastic cages under standard environmental conditions at 28 ±2 °C with a relative humidity of 75 ±10%. The mice were fed on a standard chow diet and given water ad libitum. The mice were used for experimentation after 7 days' acclimatization. All experiments were performed during the daytime. The experimental procedure was strictly in compliance with the "Declaration of Helsinki" in 1964.

Acute toxicity

Both male and female healthy mice were fasted overnight and only allowed to access to water ad libitum. They were randomly divided into five groups (10 animals per group). The mice of the first group (control group) were fed with water only. All groups were given 0.2 mL of the extract on the first day by oral gavage. The mice of groups 2 – 5 were treated with acetone extracts of *Polygonum multiflorum* Thunb. root at doses of 138, 276, 414, and 552 mg dry extract.kg^{-1} of body weight per day. The dosages were equivalent to 25, 50, 75, and 100 times the upper dosage for humans recommended in the study of **Le and Nguyen (2018)** (415 mg GAE.L^{-1}, approximately 607 mg dry extract.L^{-1} or 5.52 mg dry extract.kg^{-1} of body weight). The dosage was set at a high level to uncover any potential toxicity in order to investigate the hepatic risk. The general behavior, hazardous symptoms, and mortality of the mice were monitored for a period of 3 days after treatment. The LD_{50}, clinical biochemistry analysis, gross morphology, and histology of the liver were also evaluated in this test.

Subacute toxicity

Both male and female healthy mice were also randomly divided into four groups (10 animals per group). The mice of the first two groups (control groups) were fed with water only. The mice of groups 3 and 4 were treated with the acetone extracts of *Polygonum multiflorum* Thunb. root for 3 and 6 weeks. Treated groups were given 0.2 mL extract at a concentration of 415 mg GAE.L^{-1} (approximately 607 mg dry extract.L^{-1} or 5.52 mg dry extract.kg^{-1} of body weight, the dosage for humans recommended in the study of **Le and Nguyen (2018)**). The body weight of the mice was recorded weekly, and signs of abnormalities in the mice were recorded during the treatment period. The clinical biochemistry, gross morphology, and histology of the liver/kidney were also evaluated every 3 weeks.

Histopathologic examination, biochemical analysis, and hematological parameters

The mice were dissected to collect the livers/kidneys for histopathologic examination. Biochemical analysis was performed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), Urea/BUN (Blood Urea Nitrogen), and creatinine. In addition, a total leukocyte count and total hemocyte count were also performed.

Statistical analysis

The experimental data were analyzed by one-way analysis of variance (ANOVA) and significant differences between the means from triplicate analyses at $p < 0.05$ were determined by Fisher's least significant difference (LSD) procedure using Statgraphics software (Centurion XV). The values obtained were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Research on the gelatin-polyphenols interaction

Polyphenols concentrations of 415, 277, 208, and 166 (mg GAE.L^{-1}) react with gelatin concentrations of 30, 60, 90 and 120 mg.L^{-1} (gelatin/polyphenols ratio=1/1, v/v). The results show that relative absorbance (RA) increases with with an increase in the polyphenols concentration, and these results were significantly different (p <0.05).

The minimum and maximum RA are 0.246 and 0.718, meaning that 24.6% of the gelatin was precipitated at the minimum polyphenols concentration of 166 mg GAE.L^{-1} and the minimum gelatin concentration of 30 mg.L^{-1}. Besides, 71.8% gelatin was also precipitated at the maximum polyphenols concentration of 415 mg GAE.L^{-1} and the maximum gelatin concentration of 120 mg.L^{-1} (Figure 1). These results demonstrate that the interaction between gelatin and polyphenols is strong and significantly affects the RA. This is concordant with a study by **He, Lv and Yao (2007)**, who noted that polyphenols in tea extract interact with gelatin at a low gelatin concentration of 20 mg.L^{-1} and a polyphenols concentration of 50 mg.L^{-1} (22% of the gelatin was precipitated). The precipitate increased to 84% with an increase in the gelatin concentration (160 mg.L^{-1}) and the polyphenols concentration (150 mg.L^{-1}).

Gelatin was chosen in this study because gelatin is proline-rich, and has an open, random-coil conformation and a molecular weight of 100 kDa, thus it has a high affinity for polyphenols **(He, Lv and Yao, 2007; Frazier et al., 2010)**. Phenolic compounds can form strong hydrogen bonds easily with the protein's carboxyl group. They must be small enough to penetrate the inter-fibrillar regions of protein molecules but large enough to crosslink peptide chains at many points on the protein molecule **(Mulaudzi et al., 2012)**. The protein-polyphenols interaction depends on many factors, such as pH, temperature, the type of protein, and the structure of polyphenols **(Ozdal, Capanoglu and Altay, 2013)**.

This interaction has both advantages and disadvantages. On the one hand, it can protect the polyphenols's activity, and prevent oxidation of the surrounding environment **(Jakobek, 2015)**. On the other hand, it decreases protein quality **(Yuksel, Avci and Erdem, 2010)**, for example, by affecting protein solubility **(Rawel et al., 2002)** and by decreasing the in vitro digestion properties of proteins **(Petzke et al., 2005)**.

Acute toxicity of polyphenols extract

This extract was concentrated and was administered to each treatment group at single doses of 25, 50, 75, and 100 times the upper dosage for humans as recommended by **Le and Nguyen (2018)** (approximately 138, 276, 414, and 552 mg dry extract.kg^{-1} of body weight), respectively, by oral gavage. The control groups were treated with the same volume of distilled water (0.2 mL).

After a one-hour exposure to the extract, drowsiness and exhaustion were observed in all mice in the extract-treated group. No death or obvious clinical signs were found in any groups throughout the study. None of the extract-treated rats showed signs of toxicity in their skin, fur, eyes, sleep, salivation, diarrhea, and behavior after 72 hours. Table 1 shows that changes in clinical biochemistry analysis were not significantly different (p >0.05) at various concentrations compared with the control sample, and these results were also similar to those of other studies **(Dieu, 2009; Wu et al., 2012; Ha et al., 2015)**.

Gross morphology and histology of the liver did not show any unusual signs (Figure 2 and Figure 3). There was no difference in parenchymal tissue, portal space and central vein structures in all experimental liver sections. Hepatocytes in all experimental groups had a polygonal shape with the nucleus in the middle of the cell and were well organized in plates. The structure of the portal space was normal without inflammation, and there were no degenerative lesions in the surrounding hepatocytes. Hence, polyphenols extract from *Polygonum multiflorum* Thunb. root did not cause acute toxicity in mice, and the LD_{50} could not be estimated at the studied concentrations.

Subacute toxicity of polyphenols extract

Using a polyphenols concentration of 415 mg GAE.L^{-1} (5.52 mg dry extract.kg^{-1} of body weight), we evaluated semi-chronic toxicity over 6 weeks. After 6 weeks, all groups gained the same amount of weight, and no statistically significant differences in clinical biochemistry analysis and hematological parameters were noted between the control and treated groups at the third and sixth weeks (p <0.05) (Table 2). Gross morphology and histology of the liver/kidney did not show any injuries or unusual signs. In addition, the glomerulus had a normal structure with wide Bowman's capsules, and the renal tubes were lined by a simple cuboidal epithelium with a uniform appearance. Furthermore, no damage to the structure of hepatocytes and nephrocytes was observed (Figure 4 and Figure 5). Therefore, there was no subacute toxicity at this polyphenols concentration.

This result shows that the extract concentration employed in this study is safe for human health. Our results also differ from those of **Wu et al. (2012)**, who noticed that both acetone and water extract from fresh *Polygonum multiflorum* Thunb. roots were toxic and had dose-dependent hepatotoxicity. On the contrary, many studies reported that extract from this material is good for the liver **(Huang et al., 2007; Bhadauria, 2010)**, thus the toxicity of the extract depends on many factors, such as the extraction method, the composition of the material, solvent, etc. Moreover, toxicity was also related to the following factors: improper drug compatibility, dose, mode of administration, physical condition of the patients, and processing methods. The above-described studies appear to be contradictory. However, *Polygonum multiflorum* Thunb. roots have still been used in different ways for a long time, such as in Heshouwu tea, Heshouwu wine, Heshouwu soup, etc. in Chinese daily meals **(Li and Gao, 2015)**.

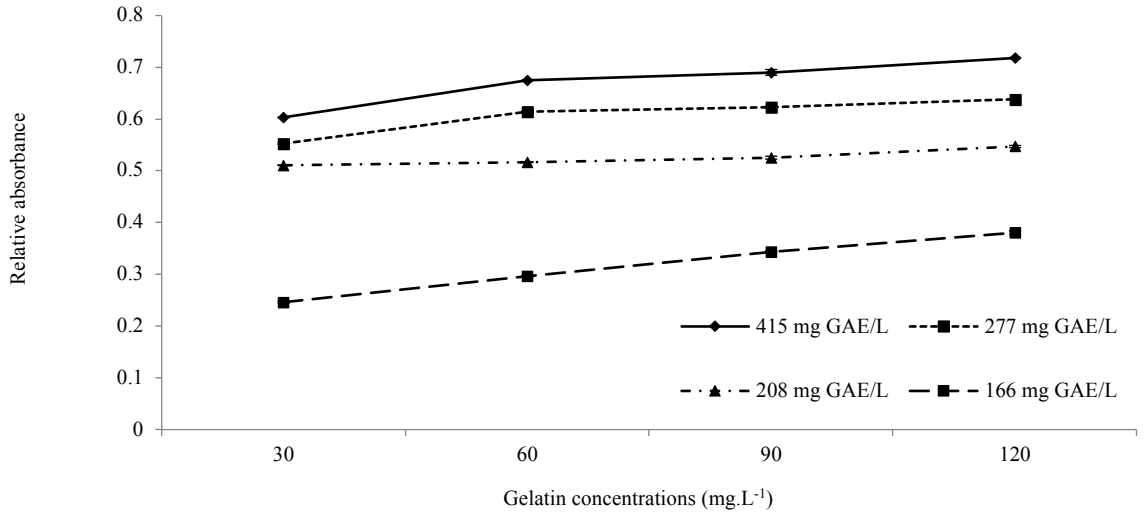

Figure 1 The RA value of the interaction between polyphenols and gelatin.

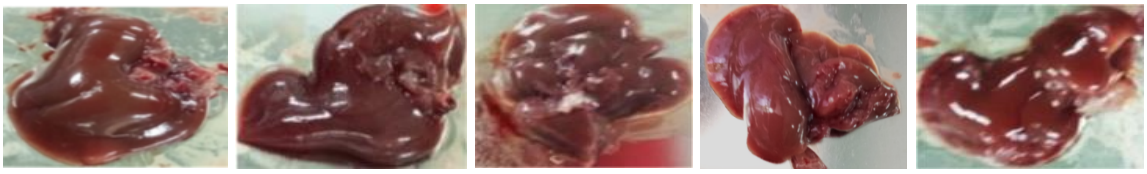

Figure 2 Gross morphology of the liver in acute toxicity**.** Note: a, b, c and d show the gross morphology of the liver at 138, 276, 414, and 552 mg dry extract per kg of body weight, respectively.

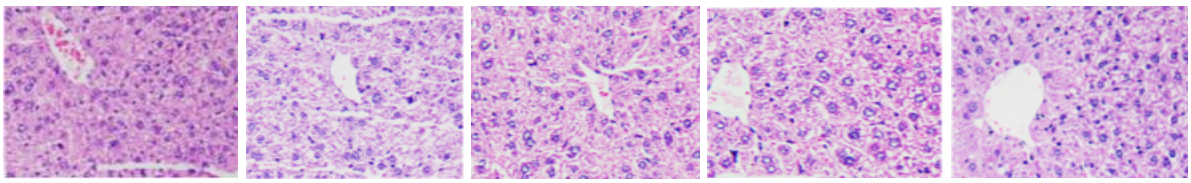

Figure 3 Histology of the liver in acute toxicity. Note: a, b, c and d show liver histology at 138, 276, 414, and 552 mg dry extract per kg of body weight, respectively.

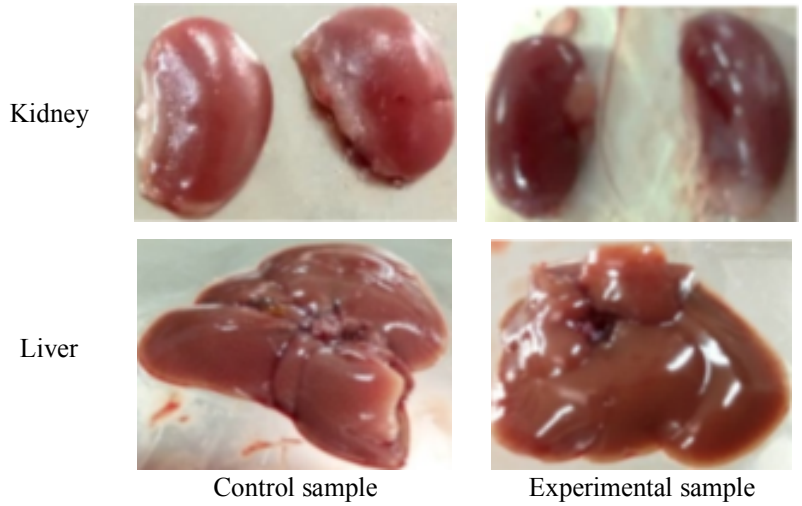

Figure 4 Gross morphology of the kidney and liver in subacute toxicity for 6 weeks.

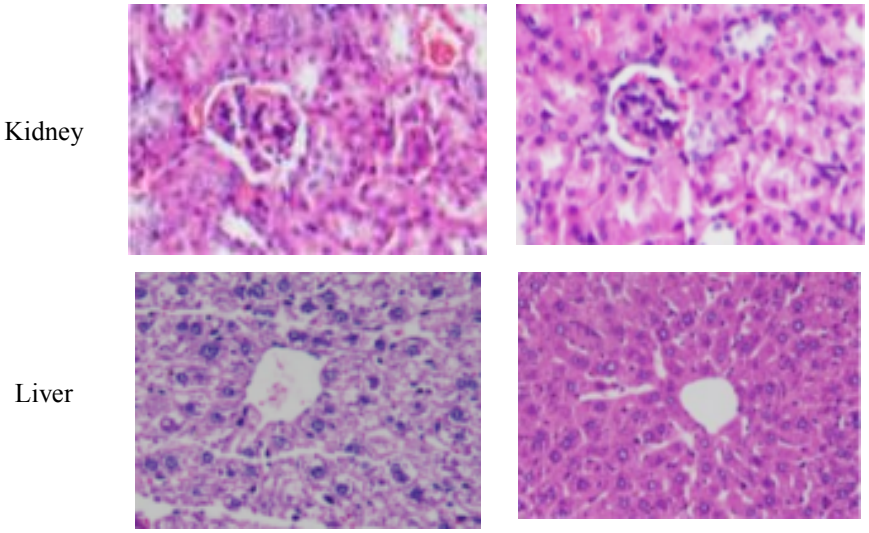

Figure 5 Histology of the kidney and liver in subacute toxicity for 6 weeks.

Table 1 Clinical biochemistry analysis and hematological parameters of mice in acute toxicity.

Criteria	Control sample	Dry extract.kg^{-1} of body weight ratio (mg.kg^{-1})			
		138	276	414	552
Urea/BUN (mmol.L^{-1})	10.51 ±1.28^{b}	8.8 ±0.75^{a}	9.03 ±1.38^{a}	9.6 ±2.22^{ab}	9.47 ±1.17^{ab}
Creatinine (µmol.L^{-1})	36.53 ±4.25^{b}	33.6 ±1.64^{a}	32.6 ±4.22^{a}	33.15 ±1.38^{a}	33.17 ±1.61^{a}
AST (SGOT) (U.L^{-1})	147.1 ±44.45^{ab}	111.43 ±25.82^{a}	173.5 ±58.03^{b}	144.1 ±52.91^{ab}	149.1 ±47.5^{ab}
ALT (SGPT) (U.L^{-1})	83.57 ±29.96^{b}	77.94 ±11.59^{ab}	61 ±7.54^{a}	82.5 ±19.05^{b}	75.3 ±12.21^{ab}
Total leukocyte count (K.µL^{-1})	1.97 ±0.25^{a}	2.43 ±0.91^{b}	2.03 ±0.15^{ab}	1.97 ±0.35^{a}	1.86 ±0.24^{a}
Total hemocyte count (M.µL^{-1})	9.33 ±0.49^{b}	8.92 ±0.57^{a}	9.2 ±0.2^{ab}	8.85 ±0.44^{a}	8.94 ±0.23^{ab}

Note: Different lowercase letters in the same row denote significant differences (p <0.05).

Table 2 Clinical biochemistry analysis and hematological parameters of mice in semi-chronic toxicity.

Criteria	3 weeks		6 weeks	
	Control samples	Experimental samples	Control samples	Experimental samples
Change in weight (%)	6.2↑	5.7↑	8↑	7.4↑
Urea/BUN (mmol.L^{-1})	6.84 ±0.72^{a}	7.36 ±1.35^{a}	8.09 ±0.71^{A}	7.2 ±1.07^{A}
Creatinine (µmol.L^{-1})	37.3 ±3.51^{a}	33.1 ±5.21^{a}	34.1 ±3.65^{A}	31 ±2.91^{A}
AST (SGOT) (U.L^{-1})	105.38 ±9.93^{a}	105.84 ±23.7^{a}	116.23 ±28.46^{A}	119.33 ±22.92^{A}
ALT (SGPT) (U.L^{-1})	62.99 ±9.09^{a}	63.67 ±13.23^{a}	70.87 ±33.46^{A}	51.27 ±8.53^{A}
Total leukocyte count (K.µL^{-1})	2.24 ±1.01^{a}	2.75 ±0.61^{a}	2.28 ±0.75^{A}	2.7 ±1.08^{A}
Total hemocyte count (M.µL^{-1})	8.17 ±1.42^{a}	8.28 ±0.93^{a}	6.21 ±1.49^{A}	7.04 ±1.68^{A}

Note: Different lowercase letters in the same row for 3 weeks denote a significant difference (p <0.05). Different uppercase letters in the same row for 6 weeks denote a significant difference (p <0.05).

CONCLUSION

In summary, polyphenols acetone extract from *Polygonum multiflorum* Thunb. roots can interact strongly with gelatin. At the same time, polyphenols extract at the studied concentrations was not acutely or subacutely toxic in mice.

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SLOVAK CONSUMERS´ PERCEPTION OF BAKERY PRODUCTS AND THEIR OFFER IN RETAILS

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ABSTRACT
Bakery products represents an important part of the diet and have an irreplaceable role in the proportion of nutrients, but their popularity in the diet of Slovak consumers continues to decline. For this reason it is necessary to address the issue of bakery products with regard to their consumption. The aim of the paper is to point out the development of consumption of bakery products in the Slovak Republic and to identify the main factors determining their purchase from the perspective of Slovak consumers. Secondary and primary data were used to meet this aim. Secondary data were obtained from the Statistical Office of the Slovak Republic in order to describe trends in consumption of selected foodstuffs in the period 2009 – 2018, as well as to predict the development of consumption of wheat and durable bread by 2020. The development of consumption is influenced by a number of factors, which may include, in particular, the price of products, the existence of substitution products, changes in eating habits and preferences of Slovak consumers, consumer health restrictions, as well as the taste factor. The primary data were obtained through consumer and business-oriented research in the Slovak Republic. The results of the consumer survey showed both rationality and irrationality in consumer decisions when buying bakery products. Rational aspects in the purchase of bakery products are factors of composition and durability. Irrationality in consumer behavior when buying can be accompanied by psychological factors, which include the perception of freshness of bakery products, the perception of prices, the perception of the country of origin, as well as sensory aspects such as taste. The achieved results were confronted with the results of a retail-oriented survey. The results showed that the commercial premises, when offering bakery products, mainly take into account the freshness, price and country of origin of selected foods.

Keywords: bakery product; consumer; factor; purchase

INTRODUCTION
The bakery industry belongs to the secondary processing industries **(Džupina, Hodinková and Kiková, 2016)**. Its raw material base are products of the food industry, which process commodities of agricultural primary production (eg mill, starch). This industry is directly influenced by the quality and price of grain, which depends on prices on world markets. The basic products of the bakery industry include durable bread, common bakery products (buns, rolls etc) and fresh bread. For most people, bakery products are a daily part of their diet. The importance of bakery products in human nutrition is significant because the products are the basis of the food pyramid and have a high nutritional value **(Al-Mussali and Al-Gahri, 2009; Nagyová, Sedliaková and Holienčinová, 2014; Kádek, 2018)**.

Hes (2009) noticed that when purchasing food, including bakery products, the consumer is influenced by the following factors. The first important aspect in the purchase of bakery products is their perceived quality and properties, which are mainly determined by sensory and health **(Stávková, Stejskal and Toufarová, 2008, Skořepa and Pícha, 2016; Wingert et al., 2014)**, and consumers are also interested in food security **(Golian et al., 2018; Adam, Hiamey and Afenyo, 2014)** and favor bakery products with higher nutritional properties (eg whole grain bread).

Nagyová et al. (2012) pointed out that consumers perceive quality on the basis of other subjective factors, such as the shape, appearance, color and taste of bread and pastry. For most consumers, the decisive factor is price **(Kubicová and Kádeková, 2011)**, which affects the purchase of specific bakery products, especially in the case of fine pastries. Another factor is the country of origin **(Kleinová and Lušňáková, 2011)**, which customers start noticing and prefer especially bakery products of Slovak origin. In the context of the country of origin, the brand is also an important factor to which consumers pay attention and choose the bakery products by specific producers or with a preferred brand name. The following factor determining the purchase of bakery products is the experience with the products, as customers prefer the bakery products with which they have a good experience. Customer automatically reaches for the bakery product he

was satisfied with and does not change his mind in the store. This situation can be described as "shopping blindness".

The recommendation can be considered as another factor positively influencing the purchase of a particular bakery product and represents an independent assessment of an unbiased person, which in some cases may have a greater impact on the customer than advertising **(Polakevičová, 2015)**. In the case of packaged bakery products, the packaging itself also affects the purchase. The packaging fulfills the communication and promotion function. The endeavor of the packaging is to attract the customer's attention, provide the customer with sufficient information about the product, its composition and thereby lead the customer to purchase. The shopping convinience and the time spent by shopping can also have a significant impact on the purchase of bakery products. The health aspect **(Šedík et al., 2019)** is another factor determining the purchase of food, including bakery products, as consumers increasingly pay attention to their health and healthy lifestyle **(Nagyová et al., 2019)** and adapt the choice of specific bakery products accordingly. In the context of the health aspect, consumers take into account the composition and nutritional value of foods **(Šedík et al., 2018)**, and in the case of bakery products they prefer whole grain ones, which are more nutritious.

The individual factors determining the purchase of bakery products can be taken into account with regard to the rationality and irrationality in consumer behavior in the choice of bakery products. **Horská et al. (2009)** combines the first approach with the rationality of consumer behavior. The purpose of this behavior is to maximize satisfaction or performance. Consumer behavior is characterized by prudence, awareness, experience, and the evaluation of alternatives **(Rovný et al., 2010)**. In the case of purchasing bakery products, consumers compare the types of bakery products, composition, method of production or prices. The second approach is linked to the irrationality of consumer decisions. **Karpińska and Krakowiak (2014)** include uncertainty, risk, limited time and access to information as factors limiting optimal purchasing choices. Consumer behavior is characterized by unpredictability, emotionality, impulsiveness or subconsciousness **(Komárková, Rymeš and Vysekalová, 1998)**.

A relatively high share of impulses and emotions is also recorded when purchasing bakery products. Purchasing decisions are influenced by several factors, including cultural, social, psychological and personal **(Géci, Nagyová and Rybanská, 2017)**. The behavior of consumers buying bakery products is mainly influenced by psychological factors, which may be decisive in re-purchasing a particular bakery product. The motivational aspect as a psychological factor may be to search for and purchase higher quality bakery products which are the basis of the food pyramid and their consumption positively affects the health of consumers due to the high nutritional value. Perception is another factor. Consumers perceive incentives and objects in bakery shops. Retailers should therefore be aware of the elements that raise or question the confidence of potential customers and should seek to understand in detail how trust affects the perception of a particular bakery product.

Personality of the consumer can also influence the choice of specific bakery products. Different types of personality choose bakery products from different perspectives, eg. depending on the type of flour, production method, aroma, color, taste. The last factor is the emotions that affect consumers, and can change depending on the experience with purchased bakery products **(Horská and Berčík, 2017; Rybanská, Nagyová and Košičiarová, 2014)**.

Scientific hypothesis

The aim of the paper is to point out the consumption of bakery products in the conditions of the Slovak Republic and to identify the main determinants affecting consumption from the perspective of the Slovak consumer. In the context of the set aim, the following hypotheses related to consumer and retails research were formulated as well.

Hypothesis 1: We assume that Slovak consumers evaluate the factors affecting the purchase and subsequent consumption of bakery products differently.

Hypothesis 2: We assume that there is a correlation between consumers' knowledge of the parbaked bakery produts in retails that are sold as the fresh baked ones and the perception of the difference in taste between parbaked and fresh baked bakery products.

Hypothesis 3: We assume that Slovak consumers perceive prices of different types of bakery products differently.

Hypothesis 4: We assume that retails assess criteria for supplier selection differently.

MATERIAL AND METHODOLOGY

The stated aim of the paper was achieved by using and processing secondary and primary data. Secondary data were obtained from the Statistical Office of the Slovak Republic. The obtained secondary data became the basis for calculating the average growth coefficient (k') and predicting the development of the consumption of bakery products by 2020 using the determination coefficient R^2.

The primary data were obtained through a consumer survey aimed at identifying key factors determining the purchase of bakery products in the Slovak Republic. The questionnaire survey was conducted on a sample of 649 respondents in the Slovak Republic in 2018, in electronic version, using the Google Forms platform. The respondents in the questionnaire survey were diversified into 8 categories in terms of gender (women 55.5%; men 44.5%), age (up to 24 years 28.4%; 25 – 39 years 20.6%; 40 – 54 years 28.4%; over 55 years 18.2%), educational attainment (basic 3.4%; secondary 57.6%; university 39.0%), permanent residence (countryside 55.3%; city 44.7%), economic status (student 29.3%; employed 44.8%; self-employed 8.0%; unemployed 3.1%; maternity leave 1.5%; pensioner 13.3%), monthly income of respondents (less than 400 € 38.4%; 401 – 800 € 32.8%; more than 801 € 28.8%), and number of household members (1 – 2 members 22.9%; 3 members 24.2%; 4 members 29.1%; 5 members and more 23.7%).

Another source of primary data was information obtained through a survey in retails. The aim of the survey was to identify the range of bakery products offer, how they approach the factors that determine consumers when purchasing bakery products, and the criteria that are

decisive in selecting a particular supplier of finished bakery products, bakery ingredients or frozen semi-finished products. The survey of retails was carried out on a sample of 107 stores in the Slovak Republic in 2018. Retails were divided according to the size of sales area (up to 400 m^2 81.3%; 400 – 2500 m^2 15.9% and more than 2500 m^2 2.8%) and geographic location (western Slovakia 72.9%; central Slovakia 14.0% and eastern Slovakia 13.1%).

Statistical analysis

The collected data were processed by using Microsoft Excel and subsequently evaluated in the statistical program XL Stat. The formulated hypotheses were tested by applying the following statistical tests:

- Chi square contingency test,
- Cramer´s coefficient,
- Friedman's test,
- Nemenyi method.

In hypothesis testing, if *p* value is lower than significant level, in case of XL Stat software by Addinsoft (version 2019.3.2), it is 0.05, the null hypothesis was rejected and alternative hypothesis was confirmed.

RESULTS AND DISCUSSION

The development of consumption of bakery products was monitored in the period 2009 – 2018. Bread consumption in the Slovak Republic in the analyzed period was at a stable level with an average annual decline of 2.1%. In the past, Slovaks could not imagine a day without a piece of bread, but at present its popularity is gradually decreasing. While approximately twenty years ago every Slovak consumed about 50 kg of bread per year, today we consume these bakery products in smaller quantities at 35 kg of bread per person per year. The trend of bread consumption development can be expressed through a linear function with the following parameters (1):

$$q_t = 41.773 - 0.8024 * t \quad (1)$$
$$R^2 = 0.9378$$

Based on the chosen linear function, it is possible to predict the evolution of bread consumption in the future, while the consumption trend is expected to decrease and in 2020 bread consumption should be at the level of 33 kg per person per year. In this context, it is important to point out the reasons for the relatively low consumption of bread, which may be affected by rising bread prices, consumer health constraints, a fairly wide assortment of plain and fine bread, a change in consumers' lifestyle, as well as various myths and falsities about bread **(Eglite and Kunkulberga, 2017)**.

Wheat bakery products consumption was slightly increasing in the period under review, ranging from 28.2 kg to 29.4 kg, which represents an average annual growth rate of 0.5%. This implies a relatively stable development of wheat bread consumption, with the highest decrease in consumption being recorded in 2013 at 27.8 kg and an increase in consumption was recorded in 2017 to the level of 30.6 kg per person per year (Figure 1**)**. We assume that by 2020 the consumption of wheat bread will slightly decrease to 29 kg per person per year. The development of the consumption of wheat bakeryproducts is positively influenced by the increasing popularity of fine pastries, among which we can include sweet buns, donuts, peelers, or modern croissants and muffins, with various fillings, toppings which feed and satisfy the appetite for sweet, at any time of the day. On the other hand, consumption is negatively influenced by several factors, which include rising prices of selected bakery products, consumer orientation towards cheaper substitutes, change of eating habits and preferences of Slovak consumers **(Kubicová and Predanocyová, 2018)**.

The last category of bakery products, the consumption of which we monitored, is durable bread. The development of durable bakery products consumption in the monitored period 2009 – 2018 was accompanied by an upward trend with an average annual growth of 1.1%. The lowest consumption of durable bakery products was recorded in 2009 and 2010, when it was 85.8 kg per person per year and the highest consumption was recorded in 2013 – 2015 and amounted to 9.8 kg per person per year. The trend of the development of the consumption of durable bakery products for the period under review can be described by the cubic function, which acquires the following parameters (2):

$$q_t = 8.1 + 0.5695 * t - 0.0731 * t^2 + 0.0029 * t^3 \quad (2)$$
$$R^2 = 0.4833$$

On the basis of the selected cubic function it is possible to predict the development of the consumption of durable bakery products in the future. By 2020, consumption of selected bakery products is expected to decline slightly to 9.5 kg per person per year. Relatively high consumption of durable bakery products can be caused by several factors, such as consumer orientation towards sweet and salty bakery products, which is readily available in stores and in packaged form, changes in preferences of Slovak consumers, taste of pastries, longer durability and the possibility of storage. The development of consumption of a given type of bread can be negatively affected by the price and wide range of substitution products **(Kubicová, 2008; Nagyová et al., 2012)**.

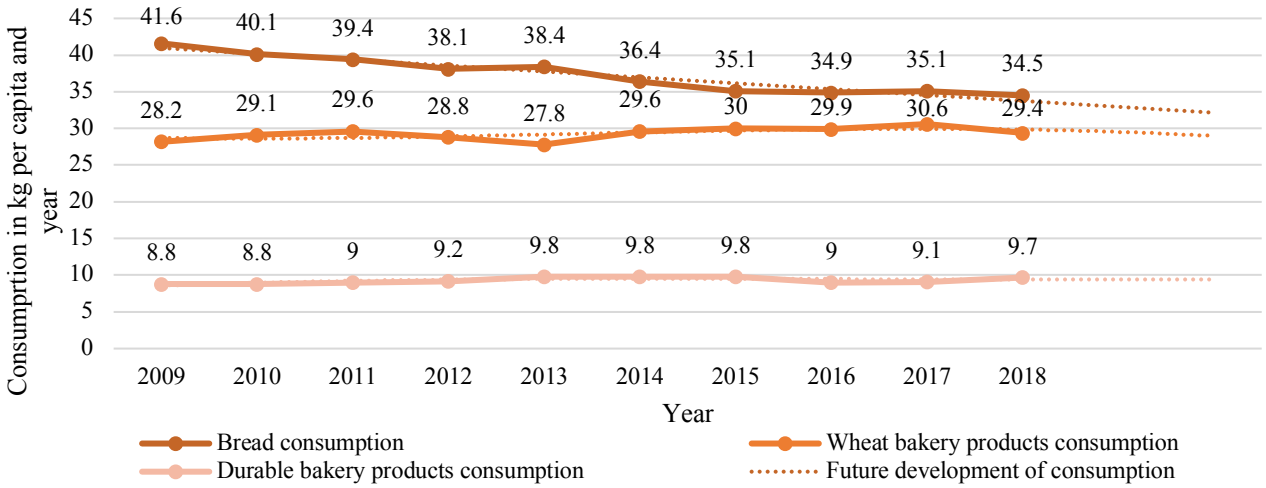

Figure 1 The development of bakery products consumption in kilograms per capita and year in the Slovak Republic. Note: **SO SR, 2019**.

Analysis of the development of consumption of individual types of bakery products showed that the consumption conditioned by the purchase of the given bakery products is influenced by a number of factors. For this reason, a questionnaire survey was carried out to identify the main factors determining the purchase and consumption of bakery products. The consumer survey for consumers of bakery products focused on the shopping behavior in the bakery products market. Based on the results of the research can be concluded that all addressed consumers buy bakery products, with the preference of fresh bakery products (99.4%) and durable bakery products (19.7%), as well as frozen bakery products that consumers bake and finish in domestic environment (18.6%). Furthermore, the obtained results show that the most preferred fresh bakery products are common bakery products such as rolls, buns etc. (82.3%), bread (70.6%) and fine pastry (35.4%).

When purchasing food, including bread, fine pastry and other bakery products, consumers make choices based on a number of criteria. We selected following ones: freshness, price, durability, composition, country of origin and taste. These were assessed by consumers involved in the questionnaire survey on the importance scale on a scale from 1 to 6, where 1 was the most important factor and 6 the least important factor. The results of the consumer survey showed (Figure 2) that the most important factors for consumers are freshness (88.9%), taste (64.3%), price (48.5%), durability (37.3%), composition (36.1%) and country of origin (25.9%). In connection with the evaluation of individual factors influencing the choice of bakery products by consumers, we found differences in the evaluation of these criteria among respondents. Based on the applied Friedman´s test, can be identified differences in factor evaluation, confirmed by a statistical calculation of the *p*-value (<0.0001), which is lower than the alpha significance level (0.05).

By using the Nemenyi method and based on the data in Table 1, we conclude that freshness is the most important criterion in the selection of bakery products (Group A), another group of important factors is flavor (Group B) followed by price (Group C). Next group of factors consisted of composition and durability (group D) and the last group of factors is the country of origin (group E). From the obtained results is possible to identify the rationality and irrationality in purchasing the selected type of food. Rational aspects in the purchase of bakery products are factors as composition and durability. The irrationality of consumers' purchasing behavior can be accompanied by psychological factors, which include the perception of freshness of bakery products, perception of the prices, perception of the country of origin, as well as sensory aspects such as taste. On a scale from 1 to 5, respondents rated the prices of bread, common bakery products (such as rolls, buns etc), fine pastry and durable bakery products, where 1 was representing very low prices and 5 very high prices. In the context of the question, we assumed that there was a difference between consumer perception of prices and the different types of bakery products and we confirmed our assumption by calculating the Friedman test (*p*-value = <0.0001). Based on the results of the questionnaire survey (Figure 4) can be concluded that most consumers perceive prices of fresh and durable bakery products as reasonable to high. **Gul et al. (2017)** found that the prices of bakery products are acceptable to consumers and consumers consider bakery products as reasonable and practice food from the point of nutritional importance. For consumers, the price perception is important from a psychological point of view. On the other hand, bread, fine pastry and common bakery products are included in the basic foodstuffs needed for nutrition of the population, which means that if the price of selected foodstuffs increases, their quantity will not change at all or only to a small extent. However, in the context of the above, it is important to note that consumers may tend to look for cheaper products within the range of bakery products, especially durable ones. The last psychological factor that is important for Slovak consumers when purchasing bakery products is the country of origin. Slovak consumers strongly perceive the country of origin when buying bakery products, especially with regard to fresh bakery products where they assume Slovak origin **(Nagyová et al, 2012)**. Consumers involved in the survey prefer the following bakery producers: Penam, a. s., Topoľčianske pekárne a cukrárne a.s., Vamex, a. s., Pekáreň Hochel, s. r. o., Lebeco, s. r. o., pekáreň Čierny

Balog, Slatinská pekáreň, s. r. o., Velapek, s. r. o., Mlyn a Pekáreň Školuda, Kamenica, Podvihorlatské pekárne a cukrárne, a. s., ERI, s. r. o., Pekáreň Gros, s. r. o., Slatinská pekáreň, s. r. o., Pekáreň ILaS, s. r. o., Trenpek, s. r. o., Gevis, s. r. o. Čachtická pekáreň, Pekáreň Beckov, s. r. o., PDP Veľké Uherce, Turpek, s. r. o., Pekáreň Juraj Oremus, s. r. o., Pekáreň Nela, Framipek, s. r. o., Prvá bratislavská pekárenská, a. s., Pekáreň Anton Antol, s. r. o., Faun, s. r. o., CPB, s. r. o., UNI, s. r. o., bakeries of retails Tesco, Billa, Lidl, Kaufland and local and regional bakeries. Gluten-free bakery products are particularly preferred from producers OLZ, Schär and JORDA´NS.

The psychological factors determining the purchase of bakery products, such as perception of freshness, perception of price and perception of the country of origin, may also be influenced by the amount of information available to consumers. Consumers obtain this information most often in stores (44.1%), where they also buy bakery products most often, and are interested in information including the composition, origin, coloring of the bread, production process, additives, production process, baking parbaked products, date of bakery production, time of baking fresh bakery products or reasons for price changes.

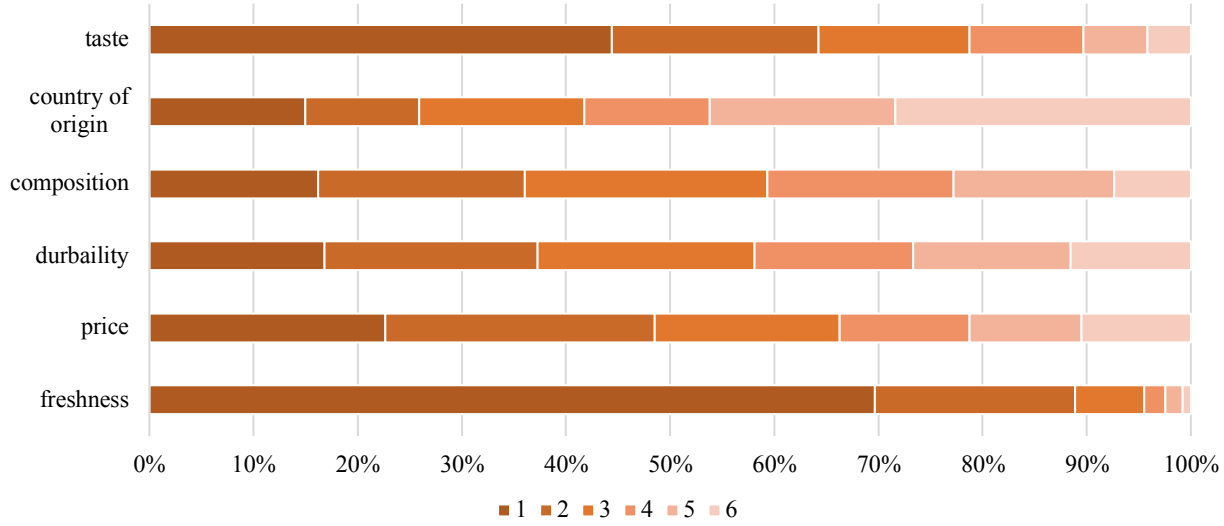

Figure 2 Factors affecting the purchase and consumption of bakery products. Note: questionnaire survey, 2018.

Table 1 Differences in factor evaluation when choosing bakery products by applying the Friedman´s Test and Nemenyi Method.

Sample	Frequency	Sum of ranks	Mean of ranks	Groups				
Freshness	649	1282.500	1.976	A				
Taste	649	1806.000	2.783		B			
Price	649	2378.000	3.664			C		
Composition	649	2571.000	3.961				D	
Durability	649	2587.000	3.986				D	
Country of origin	649	3004.500	4.629					E

Note: questionnaire survey, 2018.

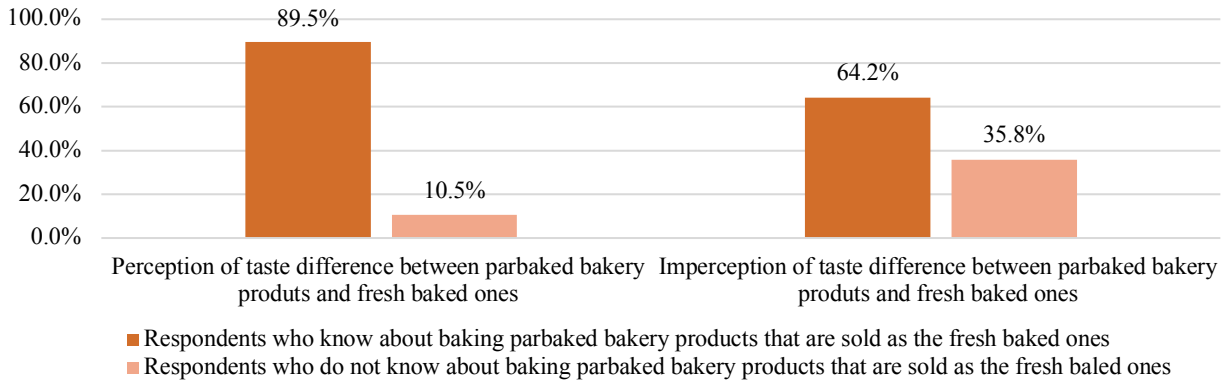

Figure 3 Perception of the difference in taste between parbaked bakery products and fresh baked ones according to consumers' knowledge of the parbaked bakery produts in retails that are sold as the fresh baked ones. Note: questionnaire survey, 2018.

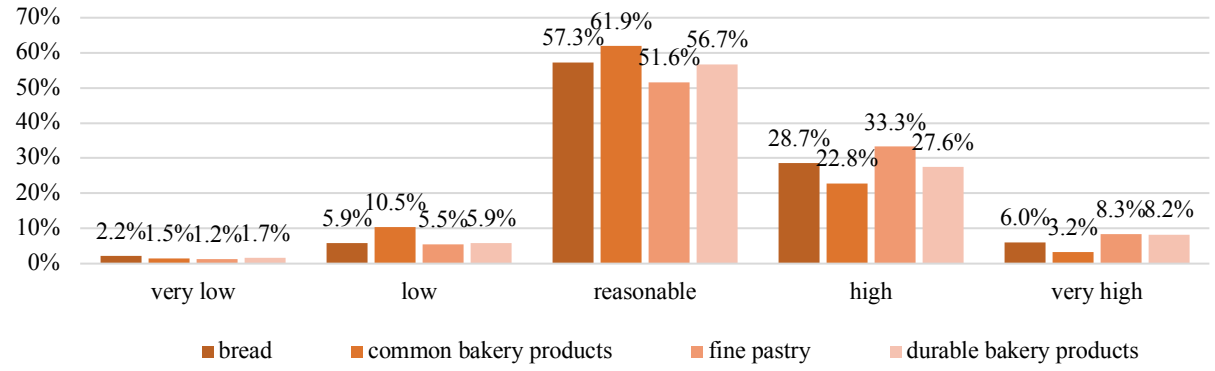

Figure 4 Perception of price of bread, common bakery products, fine pastry and durable bakery products. Note: questionnaire survey, 2018.

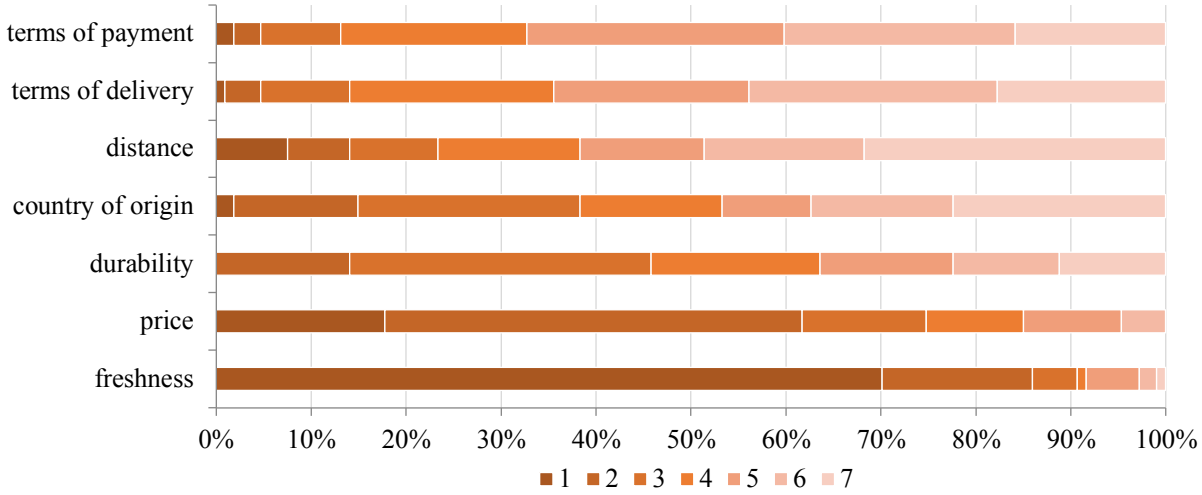

Figure 5 Factors affecting the purchase of bakery products. Note: questionnaire survey, 2018.

The obtained information then influences the perception of freshness, country of origin and prices of specifically selected bakery products by Slovak consumers.

We confronted the results of the consumer survey with the results of a retail-oriented survey. The aim of the retail survey, which also includes bakery products, was to identify the types of offered bakery products, how they approach the factors that determine consumers when purchasing bakery products and the criteria that determine the specific supplier of bakery products, bakery ingredients or frozen semi-finished products (parbaked ones). All retails involved in our survey offer bakery products, with 98.1% of the operations offering fresh bakery products, 73.8% durable bakery products and 45.8% parbaked bakery products.

As the fresh bakery products are the most preferred by consumers, we found out what kinds of these products select retails to their offer. Based on the results of the survey can be concluded that all retails offer bread, while common bakery products are available in 96.3% of retails, and 83.2% of them included in the assortment of fresh bakery products also fine pastry. Retails are trying to adapt to Slovak consumers, as fresh bread and pastries are still an essential part of the day and according to **Tovar&Predaj (2018)**, almost half of Slovak consumers who enjoy absolute freshness and crunchiness of bakery products go shopping for bakery products daily, including weekends.

Regarding the freshness, as an important factor in determining consumer purchasing behavior, we identified that 72% of the surveyed retails buy fresh bakery products from suppliers (bakers), 15% of retails buy raw ingredients from which they subsequently produce fresh bakery products and 13% of retails sell parbaked bakery products as the fresh ones. Consumer demand for fresh bakery products encourages retails to include bakery products that are warm and crunchy at the time of purchase. For this reason, they are increasingly focusing on parbaked products that are finished and baked in the store and arouse interest of customers with their smell, freshness and crispiness **(Retail Magazin, 2016)**.

As the price of bakery products is another important aspect for consumers when buying the bakery products, we were interested in factors that retails consider important in setting prices for selected types of food. Based on the results of the research can be stated that the pricing is mainly due to the prices of raw ingredients, especially wheat, which increased due to the unfavorable climate in our conditions, labor costs related to overnight work and last but not least the costs related to as fuel prices that continue to rise. In the light of the above, it can be stated that the prices of bakery products will have an increasing tendency that retails can not influence and future changes in consumer purchasing behavior are likely to be largely influenced by the amount of disposable income relative to the prices of bread and other bakery products.

The country of origin was identified as another factor determining the consumer's purchasing behavior to a significant extent, therefore we were interested how retails adapt the assortment to Slovak consumers. Research results proved that 98.1% of retails prefer Slovak producers of finished bakery products, raw materials or frozen semi-finished parbaked products. The range of fresh and durable bakery products is dominated by the following Slovak producers: Penam, Topec, Vamex, Oremus, Nella, Smatana, Pekáreň Coop Jednota, Hochel, bakery Chtelnica, Danubia, Vančo, Varipek, Labaš Nowaco, Danubia, Anton Antol, Slatinská pekáreň, Hapeko, Hopi, Duke, Bánov, Mlyn Pohronský Ruskov, Mive and smaller local bakeries. The assortment of frozen semi-finished parbaked products is provided mainly by Minit Dunajská Streda, Alfa R, Radoma Žilina and Ryba Košice, as well as by Polish suppliers. According to **Trend (2019)** and **Tovar&Predaj (2019)**, it is possible to conclude that producers of parbaked goods are doing well, as these bakery products currently account for about 30 – 40% of total baked goods consumption. In 2016, up to 43 thousand tons of parbaked bakery products were imported to Slovakia and approximately another 20 to 30 thousand tons were produced in Slovakia. Retails becomes increasingly oriented towards the purchase of parbaked bakery products, which is less costly for traders. This is also confirmed by **Tovar&Predaj (2019)** and highlights that if a supplier is the bakery, retailer have to order a certain amount of bakery products one day in advance, but the situation in operation is different every day. Accordingly, it may be the case that retail orders too much bread, which it does not sell or does not order enough. The popularity of parbaked bakery products is growing as it can provide hot bakery products throughout the whole day at the store. Retails oriented also on the sale of bakery products strive to meet the requirements of consumers, so they take into account different criteria when selecting suppliers of raw ingredients, finished bakery products and frozen semi-finished parbaked products. The selected criteria – freshness, price, durability, country of origin, distance, terms of payment and terms of delivery were ranked from 1 to 7, where 1 was the most important aspect in the selection of suppliers and 7 the least important aspect in the selection of suppliers. Research results proved that the most important criteria are freshness, price, durability and country of origin, which are also noticed by consumers when purchasing these foods. In the context of this question was established a hypothesis which assumed differences in the evaluation of the above mentioned criteria between individual retails. Based on the applied Friedman´s test, can be identified the differences in the criteria evaluation, confirmed by a statistical calculation of the *p*-value (<0.0001), which is lower than the alpha significance level (0.05).

CONCLUSION

Submitted paper focused on current issues related to the bakery industry in the Slovak Republic with a focus on the purchase and subsequent consumption of individual bakery products, as well as the identification of the main factors that determine it. We described the development trend of the bread and other bakery products consumption, which is influenced mainly by product prices, a wide range of products in retails, as well as constant changes in preferences of consumers and their purchasing behavior. The changes in consumers' eating habits are one of the most important factors affecting the consumption of selected foods. For this reason, we conducted a consumer survey. We found that the most important factors for consumers are freshness, taste, price, durability, composition and country of origin. From these factors it is possible to deduce both rationality and irrationality in the decision-making of Slovak consumers in the purchase and subsequent consumption of selected foods. Rational aspects in the purchase of bakery products are factors of composition and durability. The irrationality of consumers' purchasing behavior can be accompanied by psychological factors, which include the perception of freshness and smell of bakery products, perception of the price, perception of the country of origin, as well as sensory aspects such as taste.

Paper focused mainly on psychological factors. Consumers perceive the prices of bakery products as reasonable, which may also be due to the fact that pastries and products belong to the basic foods that the consumer is used to consuming every day. Freshness is another psychological factor that consumers perceive when purchasing the foodstuffs under review, with up to 78% of consumers being aware of the fact that retail chains fnish parbaked products in their bakeries, which are then included in the assortment of fresh bakery products. 54.5% of consumers even recognize the difference in taste between fresh bakery products and parbaked bakery products, suggesting that taste is a very important sensory aspect determining the purchase and subsequent consumption of chosen bakery products. The last identified psychological factor was the perception of the country of origin, to which consumers place a strong emphasis, as they assume Slovak origin, especially when buying fresh bakery products. We further confronted the results of the consumer survey with the results of a business-oriented survey focused on the sale of bakery products. As consumers are increasingly in demand for fresh and tasty bakery products, retail operations are encouraged to include bakery products that are warm and crunchy at the time of purchase. For this reason, they are increasingly starting to focus on parbaked bakery products that bake in their stores. Another important aspect is the price which can not be largely affected by the retials, mainly due to rising prices of raw ingredients, labor costs or transport costs. Retails offer bakery products mostly of Slovak origin due to the growing demands of consumers watching the country of origin when buying selected foods. The results also proved that the retails, when purchasing finished bakery products or bakery ingredients, take into account, in particular, the freshness, price, country of origin, durability of the bakery products as well as the distance of the suppliers.

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DIETARY FIBRE CONTENT IN ETHNIC AND UNCONVENTIONAL VEGETABLES AND FRUITS GROWING IN BANGLADESH

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ABSTRACT
Dietary fibre is known to provide health benefit and protect against degenerative chronic diseases. Thus, the present study reports the total dietary fibre (TDF) content of sixty-nine selected ethnic and unconventional vegetables and fruits growing in Bangladesh. The samples were collected from different locations of Bangladesh and mixed together to ensure sample representativeness. Dietary fibre assay kit according to the AOAC method was utilized for the analysis of TDF in selected vegetables and fruits. In the ethnic varieties, the TDF content ranged from 1.02 ±0.16 to 7.16 ±0.16 g for leafy (LV), 0.18 ±0.01 to 6.71 ±0.49 g for non-leafy vegetables (NLV) and 1.21 ±0.12 to 5.29 ±0.20 g for fruits per 100 g edible portion (EP). In the unconventional items, it arrayed from 3.08 ±0.34 g to 7.75 ±0.13 g for LV and 1.02 ±0.06 to 8.82 ±0.40 g for NLV per 100 g EP. Among the analysed samples, the highest and lowest content of TDF was found in Orohordal and Mairabokong, respectively. The unconventional vegetables contained much higher content of TDF than the ethnics and the commonly consumed similar varieties. Data on TDF content in underutilized vegetables and fruits of Bangladesh is sparse. Thus, the finding of this study would fill up the data gap in the existing food composition table of Bangladesh and also would aware the people to take vegetables and fruits rich in fibres.

Keywords: Bangladesh; dietary fibre; ethnic vegetables and fruits; unconventional vegetables

INTRODUCTION
In the recent years, health benefits of dietary fibre in reducing the risk of many chronic diseases have extensively been addressed **(Venn and Mann, 2004; Streppel et al., 2008; Aune et al., 2011; Jurasová et al., 2011)**. Epidemiological and interventional studies reported that consumption of dietary fibre rich foods such as vegetables, fruits, and whole grains reduce the blood cholesterol, especially, low-density lipoprotein and blood pressure, promote weight loss and improve insulin sensitivity **(Anderson, 2003; Streppel et al., 2008; Ivanišová et al., 2017; Rana et al., 2019)**. Diet implicates the etiology of diabetes and fibre rich diets have low glycemic index and, thus, decrease diabetic incidence **(Meyer et al., 2000; Murtaugh et al., 2003; Venn and Mann, 2004; Kaline et al., 2007; Krishnan et al., 2007)**. Dietary fibre also reduce insulin need, slow down the absorption of sugar and prevent spikes after meals **(Takekawa and Matsumoto, 2012; Kamila et al., 2018)**. Risk of cancers incidence, particularly colon cancer, has also been reported to cut to those who consume high dietary fibre containing foods **(Aune et al., 2011; Dong and Qin, 2011; Alam et al., 2017a, b)**. Intake of high fibre diets also help alleviate constipation **(Dhingra et al., 2012; Stewart and Schroeder, 2013)**. Soluble fibre has been reported to improve immunity, to speed up elimination of toxic waste through the colon and to enhance digestion. They can help treat or prevent overweight or obesity **(Takekawa and Matsumoto, 2012; Kamila et al., 2018)**.

Data on dietary fibre are sparse. Bangladesh does not yet have its own dietary fibre data; some data are being generated for a few common foods. Dietary fibre data for ethnic or unconventional foods have not yet been generated or reported elsewhere. In our present-attempt to prepare a food composition database for Bangladesh with special reference to ethnic foods **(Islam et al., 2010; Islam et al., 2012; Shajib et al., 2013; Alam et al., 2016; Alam et al., 2019; Hossain et al., 2016; Islam et al., 2016; Rana et al., 2019)**, this article reports the analysis of total dietary fibre (TDF) for sixty-nine vegetables and fruits of ethnic and unconventional varieties. Data generated in present study would surely add to and enrich the existing Food Composition Tables and database for Bangladesh **(Islam et al., 2010; Islam et al., 2012; Shaheen et al., 2014)**.

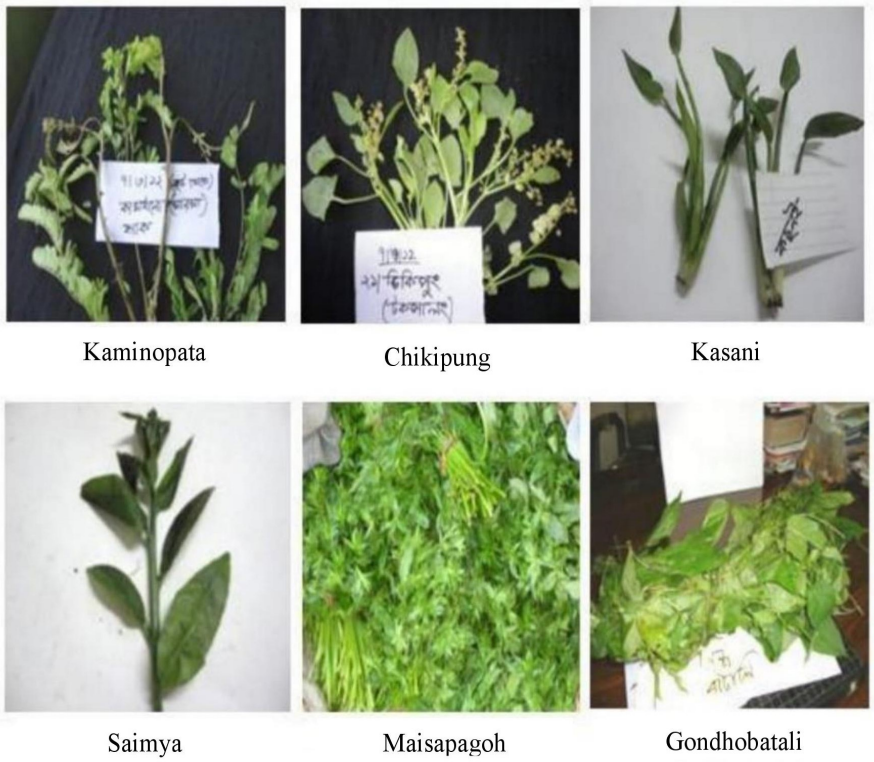

Figure 1 Photograph of the selected ethnic leafy vegetable samples studied.

Figure 2 Photograph of the selected ethnic non-leafy vegetable and fruit samples studied.

(CHTs) and in some specific plane lands. Ethnic group

Chimtishak

Venna Pata

Muktajhuri

Roktodrone

Rakhalshosha

Figure 3 Photograph of the selected unconventional vegetable samples studied.

Scientific hypothesis

The content of total dietary fibre was evaluated in different types of leafy vegetables, non-leafy vegetables and fruits consumed by specific tribal community of Bangladesh. We presumed that there exist a significant difference with respect to total dietary fibre, measured by AOAC method, in different indigenous leafy and non-leafy vegetables, as well as fruit species.

MATERIAL AND METHODOLOGY

Reagents

Total dietary fibre assay kit (TDF-100) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Reagent grade ethanol, acetone, dibasic sodium phosphate, sodium phosphate, sodium hydroxide, hydrochloric acid was procured from Merck (Darmstadt, Germany).

Food samples

This study included analysis of total dietary fibre content for sixty-nine vegetables and fruits of ethnic and unconventional varieties grown in Chittagong Hill Tracts comprised twenty-eight leafy and seventeen non-leafy vegetables and six fruits; and unconventional group consisted of fifteen leafy and three non-leafy vegetables.

Sample plan

Multi-region sampling plan was employed for collection of the food sample. To conform to the representative sample principle- "what the mass people consume' and from where they collect it"? **(Southgate and Greenfield, 2017)**, the ethnic samples were collected from weekly local markets at Rangamati and Bandarban, and the unconventional ones were collected from the specific local areas of Gazipur, Mymansingh and from some places of Dhaka, where they were grown. The samples were collected fresh, which were then water sprayed, packed into auto seal plastic poly bags and brought to the lab where the food samples were processed for analysis of dietary fibre. Depending on the availability, two to three samples were collected for each of the food from every market and growing area. These were then mixed to make three analytes or composite test samples.

Table 1a Ethnic leafy, non-leafy vegetables and fruits tested for TDF.

SN	Local Name	English Name	Scientific Name	Family
		Leafy vegetable		
1	Simeialu pata	Cassava leaves	*Manihot esculenta* Crantz.	*Euphorbiaceae*
2	Konguloaga	unavailable	Unavailable	unavailable
3	Sineiyeshak	unavailable	Unavailable	unavailable
4	Bat baittashak	Blue commelina	*Commelina benghalensis* L.	*Commelinaceae*
5	Sakumubakla	Lawn marsh	*Hydrocotyle sibthorpiodes* L.	*Araliaceae*
6	Kamino shak	unavailable	*Caesalpinia digyna* Rottler	*Caesalpiniaceae*
7	Amsurothi	unavailable	Unavailable	unavailable
8	Noyalong	Trailing Smartweed	*Ampelygonum chinense* (L.)	*Polygonaceae*
9	Monjori	unavailable	Unavailable	unavailable
10	Yangfo	Banyan Tree	*Ficus benghalensis* L.	*Moraceae*
11	Missayanu	unavailable	*Sarcochlamys pulcherrima* Gaudich	*Urticaceae*
12	Felong dal shak	Common Bean	*Phaseolus vulgaris* L.	*Fabaceae*
13	Gaiboma	unavailable	*Polycarpon prostratum* (Forssk.)	*Caryophyllaceae*
14	Chikipung	Rosy Dock	*Rumex vesicarius* L.	*Polygonaceae*
15	Ambush	unavailable	*Blumea lacera* (Burm.f.) DC.	*Asteraceae*
16	Mrolapiong	Bitter Cassava	*Manihot esculenta* Crantz	*Euphorbiaceae*
17	Projuktipata	Arrow leaf False	*Monochoria hastata* (L.) Solms.	*Pontederiaceae*
18	Khoro pata	unavailable	*Cissus repens* Lam.	*Vitaceae*
19	Katoldingi	Arum	*Lasia spinosa* (L.) Thwaites	*Araceae*
20	Kasani	False pickerelweed	*Monochoria vaginalis* (Burm.f.)	*Pontederiaceae*
21	Saimya	Lime, Sour Lime,	*Citrus aurantiifolia* (Christm.)	*Rutaceae*
22	Balapata[uc]	Pouzolzia	*Pouzolzia hirta* (Blume) Hassk.	*Urticaceae*
23	Moroi shak	Fennel	*Foeniculum vulgare* P. Mill.	*Apiaceae*
24	Kochi aampata	Mango leaf	*Mangifera indica* L.	*Anacardiaceae*
25	Dimeypata	Bitter leaves	*Glinus oppositifolius* (L.) A.D.C	*Molluginaceae*
26	Maisapagoh	Wild coriander	*Eryngium foetidum* L.	*Apiaceae*
27	Moikhumu	Edible fern	*Diplazium esculentum* (Retz.)Sw.	*Dryopteridaceae*
28	Gondhobatali	unavailable	*Paederia foetida* L.	*Rubiaceae*
		Non-Leafy vegetable		
29	Oraibalai	unavailable	*Premna esculenta* Roxb.	*Verbenaceae*
30	Shimeful	Red cotton flower	*Bombax ceiba* L.	*Bombacaceae*
31	Sengetur/seng	unavailable	*Amomum corynostachyum* Wall.	*Zingiberaceae*
32	Betagi	Canereed	*Costus speciosus* L.	*Costaceae*
33	Bas koral	Berry bamboo	*Melocanna baccifera* (Roxb.) Kurz	*Poaceae*
34	Mairabokong	unavailable	Unavailable	unavailable
35	Laigraobokong	unavailable	Unavailable	unavailable
36	Non hong he	Turmeric	*Curcuma longa* L.	*Zingiberaceae*
37	Khirar data	Cucumber stem	*Cucumis sativus* L.	*Cucurbitaceae*
38	Pudukroi	unavailable	*Amomum aromaticum* Roxb.	*Zingiberaceae*
39	Sakdusi	Crispy brinjal	*Solanum lasiocarpum* Dunal	*Solanaceae*
40	Fala	Not known	*Alpinia nigra* (Gaertn.) B.L.Burtt	*Zingiberaceae*
41	Forashdal	Kidney been	*Vigna grahamiana* L.	*Fabaceae.*
42	Kiokokro	unavailable	Unavailable	unavailable
43	Moalu	Yam	*Dioscorea bulbifera* L.	*Dioscoreaceae*
44	Rangajhumalu	Greater/water Yam	*Dioscorea alata* L.	*Dioscoreaceae*
45	Mulachi	Radish	*Raphanus sativus* L.	*Brassicaceae*

Table 1b Ethnic leafy, non-leafy vegetables and fruits tested for TDF.

SN	Local Name	English Name	Scientific Name	Family
		Leafy vegetable		
Fruits				
46	Sindire	Oriental cantaloupe	*Cucumis melo* L.	*Cucurbitaceae*
47	Rosko	unavailable	*Syzygium balsameum* (Wight)	*Myrtaceae*
48	Kushumgulo	Bead tree	*Elaeocarpus angustifolius* Blume	*Elaeocarpaceae*
49	Jogunagula	Common red stem fig	*Ficus variegata* Blume	*Moraceae*
50	Jonglikola[uc]	Bronze banana	*Musa ornata* Roxb.	*Musaceae*
51	Jongli/Bonaam	Wild mango	*Mangifera sylvatica* Roxb.	*Anacardiaceae*

Table 2 Unconventional leafy and non-leafy vegetables tested for TDF.

SN	Local Name	English Name	Scientific Name	Family
		Leafy vegetables		
52	Chimtishak	Small knotweed	*Polygonum plebeium* R. Br.	*Polygonaceae*
53	Bon palong	Bitter dock	*Rumex maritimus* L.	*Polygonaceae*
54	Tit begun shak	Black night shade	*Solanum indicum* L.	*Solanaceae*
55	Bondhonia	Wild coriander	*Scoparia dulcis* L.	*Scrophulariaceae*
56	Vennapata	Venna leaves	*Ricinus communis* L.	*Euphorbiaceae*
57	Orohorpata	Pigeon pea	*Cajanus cajan* Millsp.	*Fabaceae*
58	Bet gach	Korok bet	*Calamus tenuis* Roxb.	*Arecaceae*
59	Sornolota/ Torulota	Dodder	*Cuscuta reflexa* Roxb.	*Cuscutaceae*
60	Sadakoroi pata	Labbec tree	*Albizia procera* (Roxb.) Benth	*Fabaceae*
61	Telakucha	Ivy gourd	*Coccinia grandis* (L.) Voigt	*C urbitaceae*
62	Tetulpata	Tamarind tree	*Tamarindus indica* L.	*Fabaceae*
63	Muktajhuri	Indian acalypha	*Acalypha indica* L.	*Euphorbiaceae*
64	Khudemanik	Thankuni leaves	*Centella asiatica* (L.) Urban	*Apiaceae*
65	Roktodrone	Red verticulia	*Leonurus sibiricus* L.	*Lamiaceae*
66	Jolpai pata	Indian olive leaves	*Elaeocarpus varunua* B.	*Elaeocarpaceae*
		Non-Leafy vegetables		
67	Rakahlshosha	Wood cumber	*Zehneria scabra* (L.f.) Sond.	*Curbitaceae*
68	Orohordal	Pigeon pea	*Cajanus cajan* (L.) Millsp.	*Fabaceae*
69	Jam alu	Potato	*Solanum tuberosum* L.	*Solanaceae*

Identification of vegetable sample

A taxonomist (Dr. Maksuda Khatun) of the Department of Botany, University of Dhaka, who was also accompanied the collection team, and confirmed the sample identity with name and family. The collected samples are listed in the Table 1a, Table 1b and Table 2. Photographs of some vegetables and fruits are also provided in the Figure 1, Figure 2 and Figure 3.

Sample processing

Each of the collected samples was cleaned with tap water and then rinsed with distilled water, gently swabbed with tissue paper to remove trace of water and air dried. The air-dried sample was diced or cut into small pieces (peeled where needed) using a clean stainless steel knife on a dried clean plastic cutting board.

The diced sample was mixed, and a weighted portion was spread in stainless steel plate(s) and then dried in air-oven at 100 – 105 ˚C to constant weight **(AOAC, 2007)**, which was then milled to 0.3 to 0.5 mm mesh powder. The powdered or milled sample was stored in desiccators for analysis of total dietary fibre.

Analysis of total dietary fibre

The total dietary fibre was estimated by the enzymatic and gravimetric method of the Association of the Official Analytical Chemists **(AOAC, 2007)** using a total dietary fibre assay kit (TDF-100A, Sigma-Aldrich, Saint Louis, USA). The assay procedure as described in the kit was strictly followed. In brief, the dried meshed sample was incubated with α-amylase at pH 6.0 for 15 min at 95 ºC for gelatinization, which was then digested by incubation with protease at pH 7.5 for 30 min at 60 ºC, then with amyloglucosidase at pH 4.5 for another 30 min at 60 ºC to remove protein and starch present in the sample. Ethanol was added in excess to precipitate the soluble dietary fibre.

The residue was filtered and washed with ethanol and acetone; which was then dried overnight in an air-oven until it reduced to constant weight or nearest 0.1 mg. After

drying, half of the sample was analyzed for protein and the other half was burnt to ash.

Total dietary fibre content in the samples was calculated according to the below mentioned formula.

$$TDF\ \% = [(R_{sample} - P_{sample} - A_{sample} - Blank)/SM] \times 100$$

Where: TDF= Total Dietary Fibres, R= average residue weight (mg), P= average protein weight (mg), A= average ash weight (mg), SM= average sample weight (mg), Blank (containing only solvent)= R_{blank}- P_{blank}-A_{blank}. Residues were corrected for protein, ash and blank in final calculation.

Statistical analysis

The analysis was carried out in triplicates. Descriptive statistics were performed and values were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was employed to evaluate the differences among varieties for total dietary fibre content and was declared significant when p <0.05 at 5% level of significance. Minitab version 18.0. (Minitab Inc., State College, PA, USA) was used to analyze the data.

RESULTS AND DISCUSSION

Table 3 and Table 4 represent the total dietary fibre contents in the ethnic leafy and non-leafy vegetables and Table 5 and Table 6 represent the total dietary fibre contents in the ethnic fruits, and unconventional leafy and non-leafy vegetables. In the ethnic vegetables, the dietary fibre ranged from 1.02 ±0.16 to 7.16 ±0.16 g per 100 g fresh edible portion (pooled mean ±*SD*: 2.25 ±1.34) for leafy vegetables (Table 3), 0.18 ±0.01 to 6.71 ±0.49 g per 100 g fresh edible portion (pooled mean ±*SD*: 2.75 ±1.64) for non-leafy vegetables (Table 4) and in the ethnic fruits (Table 5), the content varied from 1.21 ±0.12 to 5.29 ±0.20 g per 100 g fresh edible portion (pooled mean ±*SD*: 3.11 ±1.44). Although the combined data for ethnic fruits showed high amount of dietary fibre compared to ethnic leafy- and non-leafy vegetables, but we did not observe any statistical significance. In unconventional vegetables (Table 6), the content ranged from 3.08 ±0.34 to 7.75 ±0.13 g per 100 g fresh edible portion (pooled mean ±*SD*: 5.79 ±1.42) for leafy vegetable and 1.02 ±0.06 to 8.82 ±0.40 g per 100 g fresh edible portion (pooled mean ±*SD*: 4.93 ±3.29) for non-leafy vegetable. Like ethnic vegetables and fruits combined data, unconventional vegetables also failed to show statistical significance between leafy and non-leafy vegetables.

Table 3 Total dietary fibre of ethnic leafy vegetables.

SN	Leafy vegetable (Local name)	TDF g per 100 g edible portion
1	Simei alu pata	1.02 ± 0.16^{k}
2	Konguloaga	2.07 ± 0.09^{de}
3	Sineiyeshak	2.36 ± 0.33^{d}
4	Bat baittashak	1.96 ± 0.15^{def}
5	Sakumubakla	4.06 ± 0.30^{c}
6	Kamino shak	1.87 ± 0.16^{defg}
7	Amsurothi	1.79 ± 0.22^{efgh}
8	Noyalong	1.74 ± 0.18^{efgh}
9	Monjori	1.12 ± 0.07^{ijk}
10	Yangfo	2.16 ± 0.10^{de}
11	Missayanu	5.06 ± 0.05^{b}
12	Felong dal shak	1.89 ± 0.12^{defg}
13	Gaiboma	1.97 ± 0.12^{def}
14	Chikipung	2.14 ± 0.15^{de}
15	Ambush	2.08 ± 0.16^{de}
16	Mrolapiong	2.41 ± 0.23^{d}
17	Projuktipata	1.67 ± 0.26^{efghij}
18	Khoro pata	1.18 ± 0.19^{ijk}
19	Katoldingi	1.68 ± 0.14^{efg}
20	Kasani	1.88 ± 0.10^{defghi}
21	Saimya	2.07 ± 0.05^{de}
22	Balapata	1.26 ± 0.18^{hijk}
23	Moroi shak	1.45 ± 0.17^{fghijk}
24	Kochi aampata	4.35 ± 0.33^{c}
25	Dimeypata	1.12 ± 0.02^{jk}
26	Maisapagoh	1.34 ± 0.04^{ghijk}
27	Moikhumu	2.09 ± 0.10^{de}
28	Gondhobatali	7.16 ± 0.16^{a}

Note: Different superscript letters in each column indicates the significant differences in the mean at p <0.05.

Table 4 Total dietary fibre of ethnic non-leafy vegetables.

SI	Non-Leafy vegetable (Local name)	TDF g per 100 g edible portion
29	Oraibalai	6.71 ±0.49^{a}
30	Shimeful	4.90 ±0.24^{b}
31	Sengetur/senga	1.70 ±0.02^{fgh}
32	Betagi	4.37 ±0.34^{b}
33	Bas koral	2.19 ±0.14^{defg}
34	Mairabokong	0.18 ±0.01^{j}
35	Laigraobokong	1.27 ±0.26^{hi}
36	Non hong he	2.98 ±0.05^{cd}
37	Khirar data	2.13 ±0.02^{efg}
38	Pudukroi	3.44 ±0.25^{c}
39	Sakdusi	2.51 ±0.47^{def}
40	Fala	2.41 ±0.32^{defg}
41	Forashdal	2.63 ±0.42^{cde}
42	Kiokokro	0.74 ±0.12^{ij}
43	Moalu	2.28 ±0.37^{defg}
44	Ranga jhum alu	1.65 ±0.13^{gh}
45	Mulachi	4.74 ±0.23^{b}

Note: Different superscript letters in each column indicates the significant differences in the mean at p <0.05.

Table 5 Total dietary fibre of ethnic fruits.

SI	Fruits (Local name)	TDF g per 100 g edible portion
46	Sindire	1.94 ±0.13^{d}
47	Rosko	4.38 ±0.46^{b}
48	Kushumgulo	1.21 ±0.12^{e}
49	Jogunagula	2.78 ±0.21^{c}
50	Jonglikola	3.06 ±0.09^{c}
51	Jongli/Bonaam	5.29 ±0.20^{a}

Note: Different superscript letters in each column indicates the significant differences in the mean at p <0.05.

Table 6 Total dietary fibre of unconventional vegetables.

SI	Leafy vegetables (Local name)	TDF g per 100 g edible portion
52	Chimtishak	7.75 ±0.13^{ab}
53	Bon palong	4.69 ±0.33^{h}
54	Tit begun shak	4.65 ±0.13^{h}
55	Bondhonia	6.27±0.33^{def}
56	Vennapata	4.49 ±0.38^{h}
57	Orohorpata	6.64 ±0.25^{cdef}
58	Bet gach	7.16 ±0.41^{bcd}
59	Sornolota/ Torulota	6.12 ±0.36^{ef}
60	Sadakoroi pata	3.08 ±0.34^{i}
61	Telakucha	7.32 ±0.34^{bc}
62	Tetulpata	6.29 ±0.16^{def}
63	Muktajhuri	6.85 ±0.46^{bcde}
64	Khudemanik	3.33 ±0.29^{i}
65	Roktodrone	6.56 ±0.11^{cdef}
66	Jolpai pata	5.74 ±0.06^{fg}
	Non-Leafy vegetables	
67	Rakahlshosha	1.02 ±0.06^{j}
68	Orohordal	8.82 ±0.40^{a}
69	Jam alu	5.19 ±0.22^{gh}

Note: Different superscript letters in each column indicates the significant differences in the mean at p <0.05.

The present study indicates that amongst the vegetables and fruits those were tested, Orohordal contains the highest amount of total dietary fibre (8.82 ±0.40 g per 100 g fresh edible portion).

It was followed by Chimtishak, Telakucha, Betgach, Gondhobatali, Muktajhuri, Orhorpata, Oraibalai, Roktodrone, Tetulpata, Bondhonia, Sornolota, which also contain rich amount of dietary fibre ranging from 7.75 ±0.13 to 6.12 ±0.36 g per 100 g fresh edible portion. The other vegetables such as Jolpaipata, Jonglaam, Jamalu, Missayanu, Shimeful, Mulachi, Bonpalong, Titbegun, Vennapata, Rosko, Betagi, Kochi aampata, Sakumubakla also contain a high amount of TDF (5.74 ±0.06 to 4.06 ±0.30 g per 100 g fresh edible portion). For leafy vegetable, the lowest TDF was present in Simeialupata (1.02 ±0.16 g per 100 g fresh edible portion). Among the fruits, Jongliaam or Bonaam has the highest amount of TDF (5.29 ±0.20 g per 100 g fresh edible portion), followed by Rosko (4.38 ±0.46 g per 100 g fresh edible portion) and Jonglikola (3.06 ±0.09 g per 100 g fresh edible portion).

The study findings also indicate that the unconventional vegetables contain higher amount of total dietary fibre (5.65 ±1.85 g per 100 g fresh edible portion) as compared to the ethnic vegetables (2.52 ±1.48 g per 100 g fresh edible portion). In case of most food items, the content of dietary fibre in ethnic vegetables was found to be, somewhat, comparable to that of commonly consumed vegetables in Bangladesh **(Islam et al., 2010; Islam et al., 2012; Shaheen et al., 2014; Alam et al., 2016; Rana et al., 2019**) as well as in India **(Longvah et al., 2017)** and elsewhere **(Dhingra et al., 2012)**. From Table 5 it can be seen that, among ethnic fruits, Rosko, which looks like Plum or Black berry, was found to contain much higher amount of dietary fibre (4.38 g per 100 g edible fresh) than the Plum (2.80 g per 100 g edible fresh) or higher than or similar to the Black berry (3.50 or 4.35 g per 100 g fresh edible portion) **(Islam et al., 2012; Longvah et al., 2017)**.

Similarly, Jongliaam and Joglikola were found to contain higher amount of dietary fibre (5.29 and 3.06 g per 100 g fresh edible portion respectively) as compared to the mango (3.65 or 1.5 g per 100 g fresh edible portion) and banana (1.90 or 2.6 g per 100 g fresh edible portion) **(Islam et al., 2010; Islam et al., 2012; Shaheen et al., 2014)**.

In the ethnic Mulachi and Green Chilli, the total dietary fibre was found to be almost same (4.74 vs 4.90 g per 100 g fresh edible portion). The recommended dietary intake (RDI) of TDF for an adult human is 30 – 38 g per day and the consumption of these ethnic and unconventional vegetables and fruits can contribute up to 30% of RDI of TDF.

CONCLUSION

Some of ethnic vegetable and fruits such as Gondhobatali, Oraibalai, Shimeful, Rosko, Betagi, Kochi aampata, Jongliaam and most of the unconventional vegetables were found to contain rich amount of total dietary fibre. The findings of present study would encourage people to adapt dietary diversity. Cultivation and regular intake of plant foods rich in fibre might reduce the risk of many diseases. It would also go a long way in filling up the data gap that exists in food composition database for Bangladesh.

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FEEDING AND WEANING PRACTICES AMONG MOTHERS OF UNDER-FIVE CHILDREN IN SELECTED PRIMARY HEALTH CARE CENTRES IN ADO-EKITI, EKITI, NIGERIA

Oluwaseyi Akpor, Tunrayo Oluwadare, Omotola Taiwo, Bukola Aladenika, Oghenerobor Akpor

ABSTRACT

An appropriate diet is necessary in the growth and health status of children especially in the first two years of life. This study determined the feeding and weaning practices among mothers of children below the age of five years in two selected Primary Healthcare Centres in Ado-Ekiti, Ekiti State, Nigeria. The study design was descriptive and cross sectional using an interviewer-administered questionnaire, 200 mothers who were purposely selected participated in the study. Statistical Package for Social Sciences was used for data analysis. Findings from the study revealed that the main practice of feeding of infants was breastfeeding, the majority of the mothers started to wean their children at about 6 – 7 months. Also, the major type of weaning practiced by mothers was abrupt weaning, majority of the mothers had good knowledge of feeding and weaning including how beneficial exclusive breastfeeding is, though it is just a few of them that practice exclusive breastfeeding. Therefore, complementary feeding education that will involve the use of various media most especially the primary health facilities is paramount for optimal health of infants. Also teaching should focus on the type of weaning and mothers should be educated on the consequences of abruptly weaning a child.

Keywords: feeding; weaning; mothers; break-feeding; complementary feeding

INTRODUCTION

Weaning is the process of gradually introducing an infant to adult foods while gradually withdrawing breast milk. The child is not abruptly taken off breast milk, the process of weaning should be started after the age of 6 months and natural weaning happens as the infant starts to accept increasing amounts and different variety of complementary feedings although still breastfeeding on request **(Mohammed, 2014; Ogunsuyi, 2016)**. Weaning is traditionally described as withdrawal from breast feeding i.e. when breast feeding is gradually replaced by semisolid food. The shift from exclusive breastfeeding to family foods is referred to as complementary feeding and complementary feeding is defined by World Health Organization (WHO) as the addition of energy as well as non-energy containing fluids, non-human milk and semi-solids or solids to children's diet which covers the time from 6 months to 18 – 24 months of age **(WHO, 2002; Chaudhry and Humayun, 2007)**.

The weaning period, which usually corresponds with the eruption of the child's major dentition implies that the child is ready to chew **(Aliyu et al., 2019)**. The weaning period is a very susceptible period, since it is the point in which malnutrition set offs in many infants. Infants are predisposed to malnutrition as a result of poor quality of weaning foods, improper feeding and weaning practices, which can also predispose the infants to growth retardation, infectious diseases and high mortality rate **(Rahul, Mohd and Rakesh, 2014; Mohammed, 2014)**.

It is indicated that poor infant feeding and their consequences are one of the world's major problems and a serious obstacle to social and economic development. During the first two years of life, poor feeding practices and weaning practices have both instant and long-standing consequences. Inappropriate feeding of infants has long been observed in our society to be one of the global problems responsible for about one-third of the cases of malnutrition worldwide **(Anoshirike et al., 2014)**. According to **Mohammed (2014)** about ten million children below the age of 5 years old die annually and more than half of the deaths occur because of malnutrition.

In Nigeria, complementary foods are usually semi-solids and they differ in homes but most homes use maize based cereal **(Aliyu et al., 2019)**. Due to introduction of westernized food and other factors like the advertising tactics taken up by most food and beverage companies and the readily preparation of most of these foods, these factors may influence the feeding practices like reducing the age of initiation of complementary feeding and early cessation of breastfeeding. Due to the high prevalence rate of malnutrition, there will be a burden on the economic

development because huge amount of money will be spent towards treating these children **(Aliyu et al., 2019)**.

Among preventive measures that would reduce the excess mortality for children under the age of five years, good quality complementary feeding, proper breastfeeding and complementary feeding practices have been listed as part and about 19% of this death can be prevented **(Kavitha, Nadhiya and Parimalavalli, 2014)**. Improved breastfeeding practices and reduction of artificial feeding is reported to save an estimated 1.5 million children a year **(Bhanderi and Choudhary, 2011)**. This study therefore investigated the feeding and weaning practices among mothers of under five children in selected Primary Health Centres in Ado-Ekiti Local Government Area of Ekiti State, Nigeria.

The theoretical framework for this study is the Health Belief Model (HBM), which is a psychological model that endeavours to describe and forecast human behaviours **(Janz and Becker, 1984)**. The HBM is an intrapersonal theory used in health promotion to design intervention and prevention programs, it is based on personal beliefs or perceptions about a disease and the schemes available to diminish its occurrence. The model proposes that people's viewpoints about health problems, perceived benefits of action and barriers to action, and self-efficacy enlightens commitment or lack of commitment in health promoting behaviour. A motivation, or cue to action must be present in order to set off the health-promoting behavior (Figure 1).

For the purpose of this study five concepts of the Health Belief Model are used. This study is based on the feeding and the weaning practices of mothers of under-five children; it involves the practices adopted by mothers to feed their children, the timing of feeding and weaning, and also the food used. Therefore, these concepts will explain how the theory is related to the study. The Health Belief Model is a framework for motivating people to take positive health actions that uses the desire to avoid a negative health consequence as the prime drive. For example, inappropriate feeding and weaning has many negative health consequences, and the desire to avoid the consequences can be used to motivate mothers into practicing safe and appropriate feeding and weaning.

Perceived severity is described as the severity an individual attach to a disease which can be as a result of the beliefs a person has about the complications that can arise as a result of the disease or its outcome on his or her health, in this case on the child's health. The main component of infant feeding is breast feeding; studies have shown that infants who are not exclusively breastfed develop some long-term medical consequences. If mothers understand the degree of health challenges that may occur due to inappropriate infant feeding and weaning, it is likely they will change for the benefit of the health of their child.

After understanding the severity of the illness that may likely occur i.e. malnutrition, mothers tend to engage in health promotion and illness prevention. And for those that are already affected they tend to find solutions before it is too late.

Perceived benefits involve one's opinion of how useful an action is in reducing the risks of developing a disease. In relation to this study, the perceived benefits include, benefits of breast-feeding, benefits of other options of infant feeding and of weaning, for example, it is

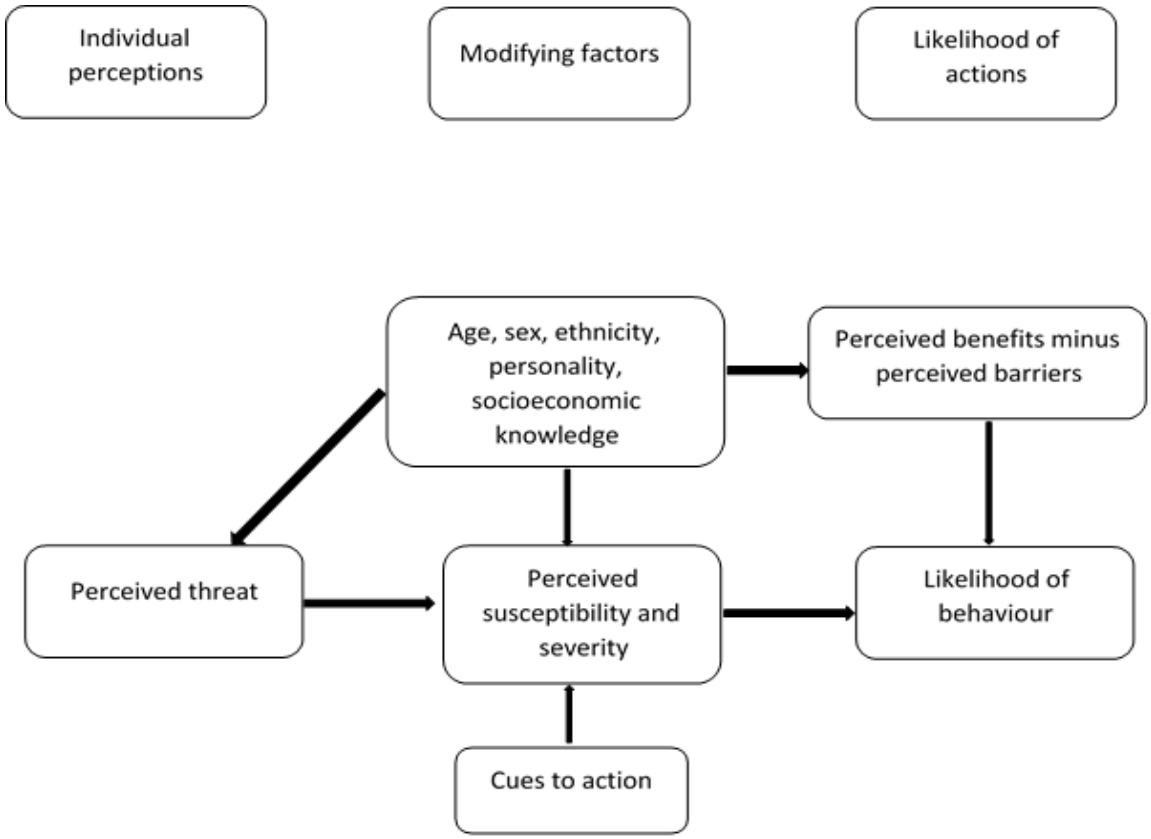

Figure 1 Diagrammatic representation of the health belief model **(Glanz, Rimer and Lewis, 2002)**.

advantageous to give thick porridge to infants above 6 months because they provide a lot of energy to the infant. Also, if a mother knows the benefit of breastfeeding for both her and her child, she is more likely to practice it. Most women do not practice the appropriate infant feeding and weaning because they are ignorant of the health risks associated, therefore, adequate enlightenment will go a long way in helping them.

Perceived barriers are the perceived obstacles that prevent an individual from taking a particular health action. It is a way of an individual assessing the barriers that will prevent him or her from adopting the new lifestyle.

There are so many obstacles encountered by women during the period of breast feeding that prevents the practice of exclusive breast feeding such as husband refusal, painful nipple, infants' refusal to suck and career. These obstacles might prevent the woman from achieving the new lifestyle, encountering difficulties with breastfeeding may cause a woman to wean her child off breast milk earlier than recommended or expected. Poverty is also a major obstacle to good weaning practices as it affects the quality and quantity of food fed to infants. Other barriers in relation to this study include, lack of education, tradition and lack of social support.

Cues to action is always essential for commitment in health promoting activities; it indicates one's willingness to assess a health facility. People who are at risk of a disease, after seeing public display on the condition tend to remember they have appointment with a medical practitioner, alarms, and reminders can also be used to keep to date. Psychological cues such as pain also serves as a reminder. The cues to action in this study include health education and counseling by the nurse. The nurse acts as a teacher, counselor and communicator to the woman which helps to promote the performance of health related behaviours such as the practice of exclusive breastfeeding.

Scientific hypothesis

Hypotheis 1: There is no significant relationship between respondents' level of education with feeding and weaning practices.

Hypothesis 2: There is significant relationship between respondents' age with feeding and weaning practices.

MATERIAL AND METHODOLOGY

Study design and setting

The study design was descriptive and cross sectional. Purposive sampling technique was used to select two Primary Health Care centres in Ado-Ekiti Local Government Area of Ekiti State, Nigeria. To maintain anonymity, the PHC were referred to as 'PHC A' and 'PHC B'.

The 'PHC A' is made up of a waiting room, examination room, labour ward and an admission ward. The services rendered in the Centre include antenatal services, delivery, circumcision, family planning, treatment of childhood diseases and immunization services. It is operated by four registered nurses and fifteen community health extension workers (CHEWS) with an average patient patronage of 744 per month.

The settings for 'PHC B' was similar to PHC A with five registered nurses and fifteen CHEWS. Services rendered in the clinic include, family planning, antenatal clinic, delivery, circumcision, treatment of childhood diseases, infant growth monitoring and immunization services. It has an average monthly patient patronage of 132.

Study population, sampling technique and sample size. The target population for this study comprised of women in the selected PHC centres whose confinement was not more than five years to avoid recall bias. Inclusion criteria included attending one of the selected PHC centres, have children below the age of five years who are being fed (bottle feeding or breastfeeding) or weaned. Potential participants had to be willing to participate in the study.

Purposive sampling technique was used to select 238 mothers that participated in the study while only 200 mothers returned fully completed questionnaire. The sample size was calculated, using the Fischer's formula for descriptive study. Although the calculated sample size of 217 and obtained, deliberate over-sampling was done to the tune of 10% (21.7) to make up for incomplete responses. Therefore, a sample size of 238 was used but only 200 questionnaires were fully completed.

Instrument and data collection

A semi-structured interviewer-administered questionnaire was used for data collection. The research instrument was developed by checking previous articles done on weaning and feeding practices and deducing questions from most of them. The questionnaire was divided into four sections, sections A and B contained the demographic data and the feeding practices applied by the participants, respectively. Section C assessed the weaning practices and foods used in weaning off the children, while Section D assessed the knowledge of mothers on feeding and weaning. The questionnaire was written in English language and participants were informed about the purpose of the study. Furthermore, pre-testing of the questionnaire was carried out using five participants that had similar characteristics with the study population but the findings were not included in the final data.

Mothers of children below the age of five were asked to participate in the study during their visit to the health care centres for ante-natal and post-natal services (booking, immunization) or family planning. Data was collected from March to April 2017. Guidelines for completion of the questionnaire was explained to the participants, questionnaires were collected and collated.

Ethical consideration

Before the commencement of the study, the research proposal was submitted to the Research and Ethics Committee of Afe Babalola University, Ado-Ekiti (ABUAD) for reviewed and permission to conduct the study was given. Likewise, an official letter was written to the two selected PHC centres and permission letters were obtained from the facility managers. Prior to data collection, each participant's right was explained and inform consent were obtained. The participants were assured that information provided will not be used against

them, no remuneration was offered and they were informed of the opportunity to withdraw at any stage of the research.

Statistic analysis

Data analysis was done with the aid of Statistical Package for Social Sciences (SPSS) version 20 and Microsoft Office Excel (2016) to generate figures and graphs. Descriptive statistics such as frequencies, percentages, mean were presented on tables, or charts.

RESULTS AND DISCUSSION

Socio-demographic characteristics of participants

With respect to the socio-demographic characteristics of participants, of the 200 participants, 50.5% of them were between the ages of 31 – 40. A total of 70.5% of the mothers were married. The vast majority (48.0%) of the mothers have 2 children, with only 1.0% of them having six children. Over 40% of the mothers had at least secondary school education (Table 1).

Feeding practices of participants

As shown in Table 2, more than half of the respondents (59.5%) fed their infants at least 6 times a day, 88.0% of the mothers breastfed their children and 63.0% did not give their children anything to drink other than breast milk in the first 3 days after delivery. Of the 37.0% of mothers who gave their infants anything other than breast milk in the first 3 days after delivery, more than half (54.1%) gave their baby glucose water.

Most mothers (60.0%) gave their children only breast milk and the minority. Almost half (42.0%) of the mothers fed their children with only breast milk till when they were 5 – 6 months, and 61.0% of the children were satisfied with only breast milk in the first 6 months.

Table 1 Socio-demographic characteristics of the participants.

Socio-demographic data		Frequency (n = 200)	%
Age distribution	20 – 30	77	38.5
	31 – 40	101	50.5
	41 – 50	22	11.0
Marital status	Single	52	26.0
	Married	141	70.5
	Divorced	7	3.5
Ethnicity	Yoruba	106	53.0
	Hausa	48	24.0
	Igbo	6	3.0
	Igbira	40	20.0
Number of children	1	35	17.5
	2	96	48.0
	3	55	27.5
	4	7	3.5
	5	5	2.5
	6	2	1.0
Educational level	No formal education	13	6.5
	Primary	34	17.0
	Secondary	83	41.5
	Tertiary	70	35.0
Occupation	Student	3	1.5
	Teacher	90	45.0
	Trader	67	33.5
	Health care worker	3	1.5
	Civil servant	27	13.5
	Unemployed	10	5.0
Age of infant	1 – 3 months	16	8.0
	4 – 6 months	40	20.0
	7 – 9 months	65	32.5
	10 – 12 months	54	27.0
	1 – 3 years	24	12.0
	4 – 6 years	1	0.5
Sex of infant	Male	87	43.5
	Female	113	56.5
Monthly income	5000	38	19.0
	6000 – 1 000	62	31.0
	11000 – 20000	52	26.0
	21000 – above	48	24.0

Table 2 Feeding practices of participants.

Feeding practice		Frequency	%
How many times do you feed your infant per day	3 Times	16	8.0
	4 Times	20	10.0
	5 Times	45	22.5
	6 Times	119	59.5
Feeding is usually?	On demand	120	60.0
	On schedule	41	20.5
	Whenever I feel like	39	19.5
Feeding method practiced	Breastfeeding	176	88.0
	Bottle feeding	8	4.0
	Spoon and cup	16	8.0
Anything to drink other than breast milk in the first 3 days after delivery?	Yes	74	37.0
	No	126	63.0
If YES, what?	Plain water	16	21.6
	Glucose water	40	54.1
	Powdered milk	12	16.2
	Infant formula	6	8.1
Which one of these milk feeding did you practice?	Breast milk only	120	60.0
	Breast milk with water	48	24.0
	Breast milk with other foods	24	12.0
	Any food available	8	4.0
How many months did you feed with only breast milk?	1 – 2 months	48	24.0
	3 – 4 months	46	23.0
	5 – 6 months	84	42.0
	6 – 7 months	22	11.0
Was your baby satisfied with breast milk in the first 6 months?	Yes	122	61.0
	No	78	39.0
How many months did you breastfeed in total?	6 months	121	60.5
	1 year	38	19.0
	1 year and above	31	15.5
	2 years and above	10	5.0
Do you practice night feeding?	Yes	162	81.0
	No	38	19.0
How frequently do you feed the child at night?	2 times	52	32.1
	3 times	41	25.3
	4 times	6	3.7
	On demand	63	38.9

Majority (60.5%) of the mothers breastfed for about 6 months, also vast majority (81.0%) of the mothers practice night feeding and close to one- third (38.9%) of the night feeding was on demand.

Weaning practices of the participants

As revealed in the study, more than one- third (39.5%) of mothers weaned their child within the age range of 6 – 7 months which is also the same age most of the mothers stopped breastfeeding their children. Close to half (44.0%) of the participants weaned their children because they had reached the weaning age, while 44.0% weaned their child off breast milk abruptly. Pap and milk (64.0%) were the food used the most by mothers during the period of weaning (Table 3).

The use of bitter substance (39.0%) was used by mothers the most as a method to take their child off breastmilk. More than half (57.5%) of the mothers gave their weaning food with a spoon and cup. A high percentage (70.0%) of the children was not selective of weaning food, 71.0% of the mothers responded that the weaning method affected the baby's weight. Majority (88.5%) of the mothers did not take drugs when they were pregnant (Table 3).

Knowledge on feeding and weaning practices

As shown in Table 4, about half (51%) of the mothers obtained their knowledge on feeding and weaning of their infants from health care workers and 82% of the mothers were familiar with the right meaning of exclusive breastfeeding (Table 4).

Almost half (51%) of the participants indicated that their knowledge on breast feeding was shown to have come from health workers (Figure 2). Generally, a vast majority (70%) of the participants were observed to have ideal knowledge of breastfeeding and weaning (Figure 3).

The majority of mothers in the study breastfed their children for at least six times daily, and breastfeeding was given for about six months in total.

Table 3 Weaning practices of participants.

Weaning practices		Frequency	%
At what age did you wean your child?	6 – 7 months	79	39.5
	8 – 9 months	66	33.0
	10 – 12 months	55	27.5
Reason for weaning child	Reached weaning age	90	45
	Not enough milk	22	11
	New pregnancy	4	2.0
	Mother/father's desire	60	30.0
	Return to work	24	12
Type of weaning practiced	Sudden	88	44.0
	Gradual	50	25
	Natural	36	18
	Mother led	26	13
Food used for weaning	Home-made food	28	14
	Ready prepared food	6	3
	Family food	82	41
	Infant formula	84	42
Type of weaning food	Pap and milk	128	64
	NAN	24	12
	Any food available	12	6
	Cerelac	14	7
	Amala	22	11
Method of weaning practiced	Use of bitter substance	78	39
	Separate mother from child	67	33.5
	Both	55	27.5
Is your baby selective of weaning food?	Yes	60	30
	No	140	70
Do you think weaning method affected baby's weight?	Yes	58	29
	No	142	71
Did you take any drug while weaning your child?	Yes	23	11.5
	No	177	88.5
If yes, mention the drug	Paracetamol	9	39.1
	Ampliclox	6	26.1
	Multi- vitamin	8	34.8

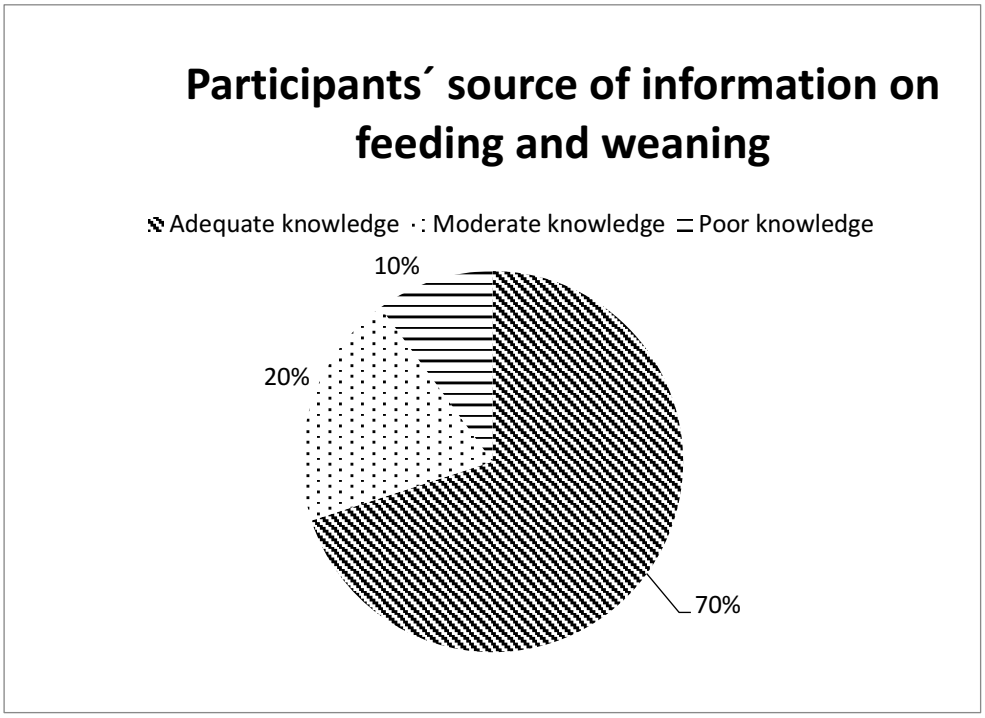

Figure 2 Participants' source of information on feeding and weaning.

Table 4 Knowledge of mothers on the feeding and weaning practices.

Items	Yes		No	
	Freq.	%	Freq.	%
Weaning is the practice of slowly introducing a baby to adult foods while slowly withdrawing breast milk	160	80.0	40	20.0
Feeding is the process of supplying food and nourishment to a baby	180	90.0	20	10.0
Exclusive breastfeeding is when only breast milk is given to a child from birth to six months of age	164	82.0	36	18.0
Exclusive breastfeeding is beneficial to the child.	156	78.0	44	22.0
Infants should be fed whenever they are hungry.	151	75.5	49	24.5
Weaning should start at 9 months.	68	34.0	132	66.0
Poor weaning practices can cause malnutrition.	164	82.0	36	18.0
Do you think colostrum is dirty, unclean?	111	55.5	89	44.5
After 6 months, it is good to only breastfeed.	37	18.5	163	81.5
Young children should be breastfed at least 2 years?	94	47.0	106	53.0
Infant formula is as good as breast milk.	72	36.0	128	64.0

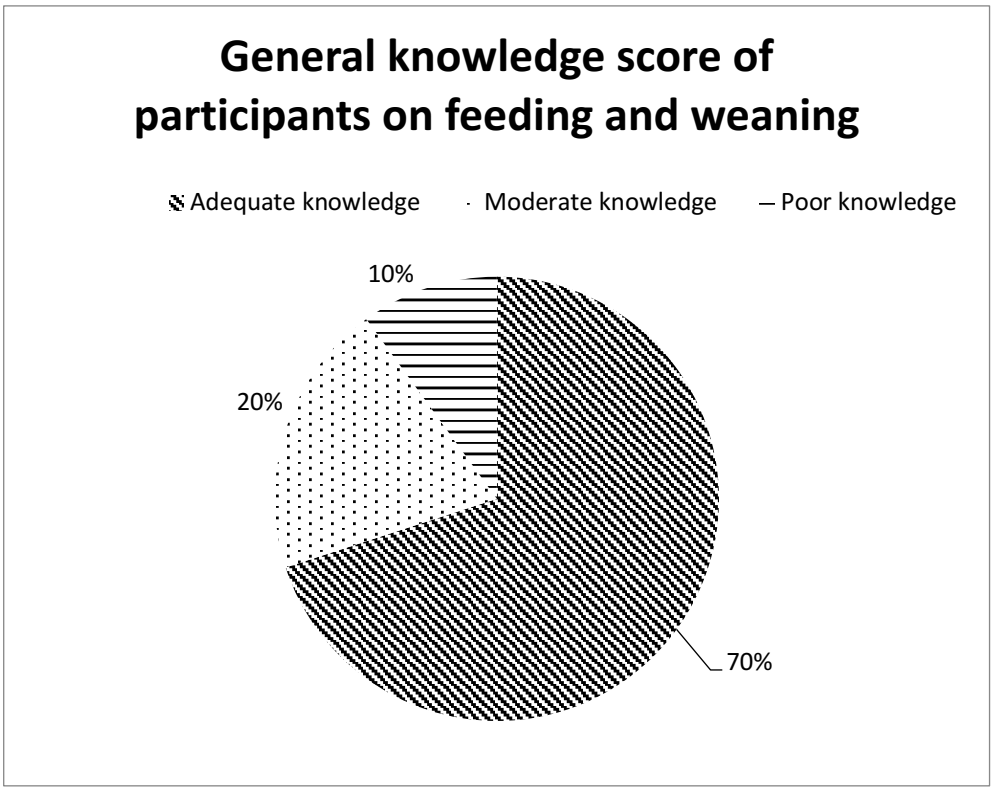

Figure 3 General knowledge score of participants on feeding and weaning.

Table 5 Relation of respondents' educational status with feeding and weaning practices.

		Practice grading			Total
		Adequate practice	Moderate practice	Poor practice	
Level of education	No formal education	13	0	0	13
	Primary	18	16	0	34
	Secondary	59	24	0	83
	Tertiary	30	20	20	70
Total		120	60	20	200

Table 6 Chi-Square Tests.

	Value	Df	*p*-value
Pearson Chi-Square	53.298[a]	6	.000
Likelihood Ratio	61.272	6	.000
Linear-by-Linear Association	21.628	1	.000
N of Valid Cases	200		

Note: [a] – *p*-value <0.05.

Table 7 Relationship of respondents' age with feeding and weaning practices.

		Practice grading			Total
		Adequate practice	Moderate practice	Poor practice	
Age	20 – 30	35	27	15	77
	31 – 40	73	28	0	101
	41 – 50	12	5	5	22
Total		120	60	20	200

Table 8 Chi-Square Tests.

	Value	Df	*p*-value
Pearson Chi-Square	27.167[a]	4	.000
Likelihood Ratio	34.900	4	.000
Linear-by-Linear Association	5.543	1	.019
N of Valid Cases	200		

Note: [a] – *p*-value <0.05.

This study agreed with the findings of **Akeredolu et al. (2014)** and **Olatona, Odozi and Amu (2014)**, who asserted in their studies that majority of the mother's breastfed their children. In a related study, **Katepa-Bwalya et al. (2015)**, it was also observed that majority of mothers breastfed their children for varying periods of time. As revealed in the study, of the majority that practiced breastfeeding, only few (14.7%) breastfed exclusively. This is similar to the findings of **Mohammed (2014)** and **Katepa-Bwalya et al. (2015)**, where only 30.1% and 6.8% of mothers practiced exclusive breastfeeding respectively. Despite the fact that breast feeding is known to be a highly recommended and beneficial way to feed infants as it provides psychological and health benefit to both the mother and the child. Globally there has been a common decline in the practice of breastfeeding both in terms of occurrence and interval in the past few decades **(Berra, 2013)**.

The study further revealed that about 40.0% of the mothers in addition to breastfeeding included complementary foods for their children at 4 – 6 months of age. This contrast with the results obtained from some studies conducted in South-West, South-South and North-West regions of Nigeria where 76.7%, 79.9% and 80.1% respectively introduced complementary feeding before the expected 6 months of age **(Akeredolu et al., 2014; Osie-Efetie, Oyibo and Okperi, 2011; Matthew et al., 2009)**.

When breast milk is no longer adequate to meet the nutritional needs of the infant, complementary foods should be added to the diet of the child **(Kavitha, Nadhiya and Parimalavalli, 2014)**. Proper breastfeeding practices are effective ways for reducing childhood morbidity and mortality. While many mothers understand the significance of breastfeeding, others are less informed on the benefits of breastfeeding and weaning. **Anoshirike et al. (2014)** stated that, malnutrition in Nigerian infants is found to be as a result of inappropriate child feeding practices such as late introduction of complementary foods, small energy and nutrient density of foods offered, feeding in little quantity at meals, food limitations due to cultural beliefs, low birth-weight and high morbidity.

Hypothesis testing

Hypothesis 1:

From the chi-square test (Table 6), the null hypothesis of no significant relationship between respondents' level of education and deeding and weaning practices is rejected. Consequently, the alternative hypothesis of a significant

relationship between respondents' level of education with feeding and weaning practice accepted (Table 5).

Hypothesis 2:

As shown in Table 2, there was no significant relationship between respondents' age and feeding and weaning practices, hence the null hypothesis of a significant relationship between respondents' age with feeding and weaning practices was rejected (Table 7 and Table 8).

Adequate nutrition during infancy and early childhood is essential to ensure the development, health, and growth of children to their full potential. As stated by **Mohammed (2014)**, gradual weaning period should be introduced first which allows the child to receive the benefits from breastfeeding while also consuming necessary nutrients from complementary foods. This is contracts with the result obtained in this study where about 45% of mothers abruptly weaned their children from breast milk. According to **Razia and Naheed (2007)**, complete weaning can be introduced at about 2 – 4 years of age, and this is the total withdrawal from breast milk. The findings of the study showed that 39.5% of the mothers weaned their children at about 6 – 7 months and 45% of mothers weaned because they were of the opinion that the child is old enough. This is similar to the results obtained by **Kikafunda, Walker and Tumwine (2003)**, **Mohammed (2014)**, **Olatona, Odozi and Amu (2014)** and **Ogunsuyi (2016)**, where 42%, 91.6%. 48.4% and 55.6% respectively started the weaning process between ages 4 – 6 months. Feeding and weaning are important components for the physical and psychological well-being of a child. Feeding a child which involves breast feeding is a complex approach while weaning is a critical approach. According to **Kavitha, Nadhiya and Parimalavalli (2014)**, displacement of breast milk and increased risks of infections such as diarrhoea which further contributes to weight loss and malnutrition can be as a result of untimely introduction of complementary feeds before the age of six months. Thus, it is important that weaning should be gradual and not done abruptly. In addition, the findings revealed that during weaning, homemade food, infant formula and family food were given to the infants by the mothers.

The present study revealed that the majority of participants had good knowledge (70%) on feeding and weaning of their infants, with most indicating their knowledge came from the information provided by health workers. Poor infant feeding and their consequences are one of the world's major problems and a serious obstacle to social and economic development, therefore adequate knowledge on the feeding and weaning practices of the children is very essential. Nurses can assess the knowledge of mothers attending post-natal clinic towards feeding and weaning, certain educational programmes can be conducted for the mothers based on the needs which will help improve the mother's knowledge regarding feeding and weaning. Since health care workers serve as a strong means of disseminating health information to the community; they too need to be abreast of information **(Kambli, 2012)**.

CONCLUSION

This study assessed mother's feeding practices, their weaning practices and also their knowledge on the feeing and weaning practices. The findings from the result showed that the vast majority of the mothers have good knowledge about feeding and weaning of their infants.

The practices of mothers on the feeding of their children were good based on the fact that breastfeeding was the major practice by mothers and also the knowledge of mothers on feeding and weaning their children was good. Specifically, the results of this study provide a baseline data on the importance of establishing standard indigenous nutrition education programme for mothers of young children in Nigeria. Emphasis should be placed on including pregnant women and women of child bearing age as the target audience. This will facilitate the mothers understanding of nutrition which will in turn enhance the feeding practices and nutritional status of their children. There is need concerning health education program aimed at educating mothers on commencement of breastfeeding within one hour after birth, exclusive breastfeeding till six months of age and the value of night feed and the benefits of giving infants colostrum.

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AUTHENTICATION OF WISTAR RAT FATS WITH GAS CHROMATOGRAPHY MASS SPECTOMETRY COMBINED BY CHEMOMETRICS

Any Guntarti, Ibnu Gholib Gandjar, Nadia Miftahul Jannah

ABSTRACT
Indonesia is a country with the largest Muslim population in the world, which is very concerned about halal food. The most problem that's very concerning nowadays was that food products were contaminated by unclean meat, such as rat meat. The purpose of this study was to authenticate rat fat using Gas Chromatography-Mass Spectrophotometry (GC-MS) combined with chemometrics. In this study, rat fat were heated in oven at 90 °C – 100 °C for approximately one hour until the oil came out. After that, the derivatization process was carried out to convert fat into methyl ester compounds using NaOCH3 and BF3. Methyl ester compound than injected into the GCMS instrument system. In addition to rat fat, other fat extraction were carried out, such as pigs, cows, chickens, wild boars, dogs, and goats. The combination of chemometrics Principal Component Analysis (PCA) was used to classify rat fat with other animal fat. Based on the results of the study showed that fatty acids in rats using GCMS produced 6 types of fatty acids, namely: myristat (0.15 ±0.09%), palmitoleate (0.73 ±0.54%), palmitate (19.08 ±3.54%), linoleate (30.14 ±16.90%), oleate (40.48 ±2.74%), and stearate (2.55 ±0.01%). Total content of rat fatty acids was 93.13%, with unsaturated fatty acids 71.35% and saturated fatty acids 21.78%. Chemometrics PCA from rat fat can be grouped with other animal fats.

Keywords: chemometrics; food; GC-MS; halal; PCA; Wistar rat fat

INTRODUCTION
Food is a basic human need, therefore food availability needs serious attention both in quality and quantity. Indonesia is a country with a Muslim majority of 207.2 million with a presentation of 87.18% in 2010 out of a total population of 237 million **(Muslim and Purwanto, 2013)**. In addition to food safety factors, the halal factor of a food product must also be of concern to the Muslim community. At present the awareness of the Muslim community to consume halal food increases along with the awareness of the Muslim community following Islamic laws **(Rohman et al., 2016)**. Along with the increase in people's income, the demand for meat consumption in various regions of Indonesia has increased. The price of basic ingredients which are quite expensive such as chicken meat, makes many producers mix it with meat which is relatively cheaper, one possibility is to use rat meat **(Guntarti and Prativi, 2017)**. Rat meat is a meat that is quite easy to obtain, even it can be obtained free of charge. Some media also reported the adulteration of beef meatballs with rat meat **(Lumakso et al., 2015)**. Examples of several cases on the market are forgery of chicken nuggets from pork, and nuggets from recycled materials **(Sari and Guntarti, 2018)**. Based on this, it is also feared that counterfeiting of processed chicken products using rat meat will also occur in Yogyakarta. Laboratory tests to determine fatty acid markers in the form of methyl esters in rats include using gas chromatography-gas spectrophotometry combined with chemometrics. This technique has been used in a variety of analyzes, such as food and pharmaceutical products **(Ronggo et al., 2007)**.

The chemometric method is one way to obtain important information about certain objects in the data by using statistical or mathematical techniques. The most commonly used types of chemometrics are (1) grouping techniques, such as Principle Component Analysis (PCA) and (2) quantitative analysis techniques with multivariate calibration, such as Partial Least Square (PLS).

Scientific hypothesis
The hypothesis in this study is that methyl esters from wistar rat animal fat can be analyzed using the Gas Chromatography Mass Spectrometry (GCMS) method. The methyl ester data combined with chemometrics is able to classify types of fat.

MATERIAL AND METHODOLOGY
Fat samples
Samples in the form of pork, beef, dog, goat, wild boar, chicken were obtained from the traditional market, Wistar white rats were obtained from other researchers' carcasses, the fat was taken. Materials used *n*-hexane (technical),

$NaOCH_3$ 0.2 N solution (E-Merck, pro-analysis quality), BF_3 solution (E-Merck, pro-analysis quality), saturated NaCl and anhydrous Na_2SO_4 (E-Merck, pro-analysis quality) **(Rohman and Che Man, 2011; Kumar et al., 2014)**.

Tools

GC-MS distributor from Ditek Jaya, Merck Shimadzu, Japan, type GCMS-QP2010 SE. The column used was Rtx-5ms, DB1-MS Restech, 30 m x 0.25 mm ID, 0.25 µm, stationary phase of polymethyl xiloxan, injector temperature of 230 °C, column temperature of 70 °C and increased to 300 °C with an increase of 10 °C.min^{-1}, and flow rate of 1.15 mL.min^{-1}. The mobile phase of Helium gas. MS Detector *Electron Multifier Detector* (EMD) 70 MeV. The mass spectrum was compared to the WILLEY147 & NIST47 library found in the GC-MS software. The methyl ester was injected as much as µL into the GC column in a manner of autosampler.

The Research Progress

Fat Extraction

Intake of fat was done by rendering at a temperature of 90 – 100 °C for approximately one hour in the oven. The resulting fat was then added with anhydrous Na_2SO_4 and then centrifuged at 3000 rpm for 20 minutes **(Rohman et al., 2012)**. The solution was stored in the refrigerator at -20 °C in a rolling test tube. The solution was used for the derivatization process.

Derivatization

Fat derivatization aims to convert fat into a form of fatty acid methyl ester by using NaOCH3 0.2 N and BF_3 solution. Derivative products containing fatty acid methyl ester derivatives (FAME) were taken and injected into the gas chromatograph system. A total of 1µL supernatant was injected into gas chromatography-mass spectrometry, replicated two times.

Statistical analysis

The results of the analysis in the form of a mass spectrum were compared with the WILLEY147 & NIST47 libraries contained in the GC-MS software. Data obtained from GC-MS was fatty acids in the form of methyl esters. The content of methyl esters of fatty acids from each animal fat was grouped using chemometrics PCA with minitab 16.

RESULTS AND DISCUSSION

Fat Extraction

Rendering extraction to obtain rat fat. The advantages of this method are that there are many extract yields, easy and inexpensive processing because it does not involve chemicals **(Rohman et al., 2016)**. The yield of 16.36%. The yields are influenced by the intake of food from the rats themselves, body fat taken, and the way the fat is extracted **(Lobb and Chow, 2007)**. The obtained lipid fraction was then carried out by the esterification process. The esterification reaction aims to convert the fatty acids into their methyl ester forms. The esterification process is carried out using BF_3 as a catalyst. BF_3 is an acidic compound **(Purbasari and Silviana, 2008)**. Figure 1 is a process of the esterification mechanism and the product of methyl ester is obtained.

Fatty Acid Composition in Wistar Rat.

Analysis of methyl esters from Wistar rat derivatization using gas chromatography-mass spectrometry (GC-MS) method. This instrument is a combination of gas chromatography with a mass spectrometer detector. Gas chromatography is used for the separation of fatty acid content in the form of methyl esters. In addition to the retention time (tR) of the separation results in gas chromatography, there is similarity index (SI) information to determine the proximity of the chemical structure of the type of fatty acids. The result of SI >90 shows the similarity of mass ion overflow to the target spectra/fat sample.

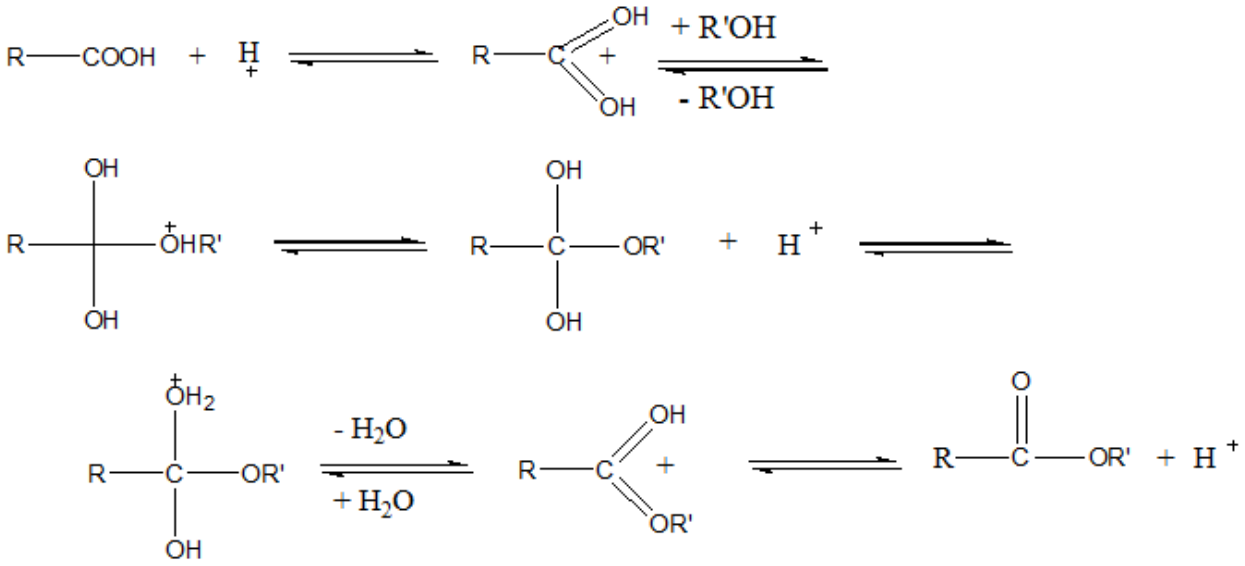

Figure 1 The Process of Esterification Reaction Mechanisms with Methanol and using Acid Catalysts (adapted from **Purbasari dan Silviana, 2008)**.

Table 1 Separation results and identification of compounds in Wistar rat fat with GC-MS.

Number	t_R (min)	% Area (x ±*SD*) (n = 2)	SI	MW	Compound Name
1	17.75	0.15 ±0.09	95	242	(C14:0) Methyl Myristate
2	20.34	0.73 ±0.54	96	268	(C16:1) Methyl Palmitoleate
3	20.81	19.08 ±3.54	96	270	(C16:0) Methyl Palmitate
4	25.60	30.14 ±16.90	86	284	(C18:2) Methyl oktadekadienoat/linoleate
5	25.93	40.48 ±2.74	88	296	(C18:1) Methyil Oleate
6	26.51	2.55 ±0.01	97	298	(C18:0) Methyl Stearate

Note: SI= *Similarity Index*, MW= Molecular weight.

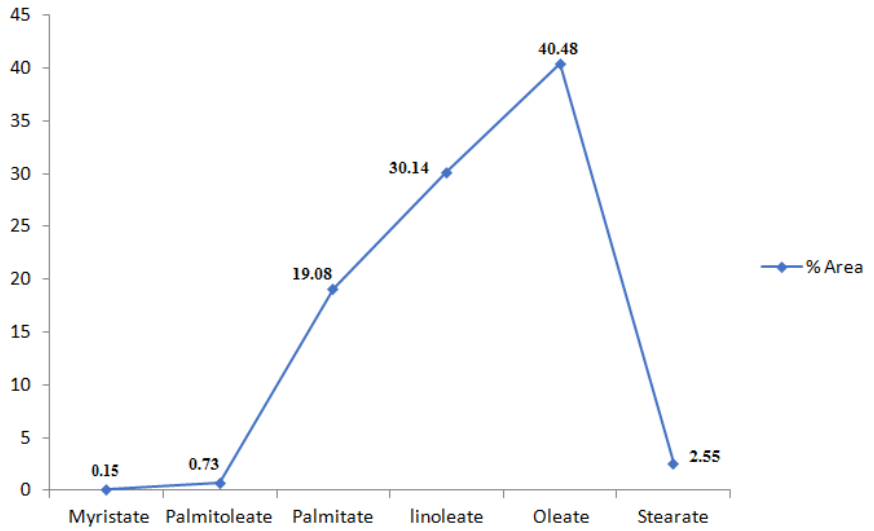

Figure 2 Line of the type of fatty acids in Wistar rat fat.

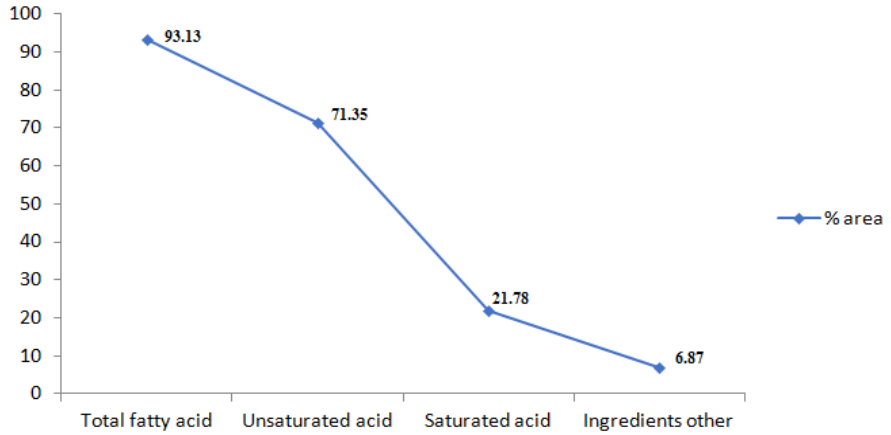

Figure 3 The Line of total fatty acid, saturated fatty acid, unsaturated fatty acid, and ingredients other content in Wistar rat.

Table 1 presents retention time (tR), % peak area, SI, molecular weight (MW) and estimated compounds and identification of white rat fat **(Guntarti and Amidin, 2018)**. The results of GC-MS analysis in Table I show the results of SI values >90 except oleic acid which is 88% and linoleic acid 86%. This shows that the target fatty acid type is suitable or similar to the comparison spectra. The fatty acid compound with a tR of 25.93 minutes and a SI value of 96% was similar to the comparison compound with the formula $C_{17}H_{36}O_2$ with m/z 296. The fatty acid was in the form of its methyl ester. Whereas if in the form of fatty acids, the compound formula is $C_{18}H_{33}O_2$.

Fat is an unstable component in the presence of light. The results of the analysis with GC-MS showed that oleic acid was the highest constituent component of fatty acids in rat fat with a percentage of 40.48%, followed by linoleic acid 30.14%, palmitic acid 19.08%, stearic acid 2.55%, palmitoleic acid 0.73%, and myristic acid 0.15%. The line of the types of fatty acids in Wistar rat fat is presented in Figure 2. unsaturated fatty acid with one double bond.

When viewed from unsaturated bonds, white rat fat contains many types of unsaturated fatty acids, namely palmitoleic acid (0.73%), linoleic acid (30.14%), and oleic acid (40.48%). Whereas saturated fatty acids are myristic acid (0.15%), palmitic acid (19.08%), and stearic acid (2.55%). If looked at the percentage of the content, then more unsaturated fatty acids is equal to 71.35%. Saturated fatty acids of 21.78% and 6.87% are ingredients other than methyl esters. Larger amount of unsaturated fatty acid content will affect the physical form of fat at room temperature and the stability of fat. Figure 3 presents a line of total fatty acid, saturated fatty acid, unsaturated fatty acid, and ingredients other content in Wistar rat.

Comparison of fat: Wistar rat, dog, wild boar, beef, pork, chick and goat.

Besides Wistar rat fat, other animals' fat used were: dog, beef, pork, chick, and goat. Fat retrieval is the same as done in white fat retrieval, which is by rendering with an oven at a temperature of 90 °C – 100 °C, for 30 – 60 minutes. The fat obtained is esterified to form its methyl ester with $NaOCH_3$ and BF_3 which are then injected into the GC-MS system. The results of analysis of dog, beef, pork, chick, goat, and wild boar fat are presented in Table II and Figure 4.

Based on Table II it can be seen that the fat of white rat and pork containing linoleic content (30.14%), and pork fat (21.49%). The highest oleic acid is in the content of pork (55.66%). Goat fat has the highest type of saturated fat, palmitate (23.55%) and stearic (47.13%).

In the results of previous studies **(Hermanto, Muawanah and Harahap, 2008)**, that pork and chicken fat contain margaric acid. Margaric acid content (C17: 0) in pork is 0.5%, and in chick 1.74% **(Hermanto, Muawanah and Harahap, 2008)**. **Guntarti (2018)** research results: beef fat has a high stearic acid (35.03%), while oleic acid is 14.90%. The results of this study, beef fat has a high oleic acid content (52.29%), while stearic acid is 12.59%. Except for goat fat, all animals contain the highest oleic acid; while goat fat, the highest is stearic acid. Figure 4 presents the content of saturated and unsaturated fat, and the total amount of fatty acids in various animals. Based on Figure 4, the saturated fatty acid content is high in goat fat (71.16%), wild boar fat (36.27%), beef fat (35.16%), dog fat (34.33%), lard/pork (27.78%), and the smallest is in chick fat (20.55%). The highest unsaturated fat content is in chick fat (73.2%), rat fat (71.35%), lard / pork fat (55.66%), dog fat (53.93%), wild boar fat (45.24%), and the smallest is in goat fat (19.19%). While the highest amount of total fat is in chick, followed by white rat.

Table 2 The results of the analysis of acid content in the fat of: Wistar rat, dog, wild boar, pork, chick, beef, and goat with GC-MS.

Methyl ester	Percentage of (%) methyl esters						
	Dog	Wild boar	Pork	Chick	Beef	Goat	White rat
Methyl myristat (C14:0)	0.33	nd	0.41	nd	0.29	0.25	0.15
Methyl pentadekanoate (C15:0)	nd	nd	nd	nd	0.36	0.23	nd
Methyl palmitoleic (C16:1)	0.34	nd	1.14	1.14	0.98	nd	0.73
Methyl palmitate (C16:0)	16.42	19.65	17.26	18.91	21.81	23.55	19.08
Methyl margarate (C17:0)	0.37	0.27	nd	nd	0.11	nd	nd
Methyl linoleate (C18:2)	nd	nd	25.75	21.40	nd	nd	30.14
Methyl oleate (C18:1)	53.59	45.24	55.66	50.66	52.29	19.19	40.48
Methyl stearate (C18:0)	17.21	14.37	10.11	1.64	12.59	47.13	2.55

Note: nd= not detected.

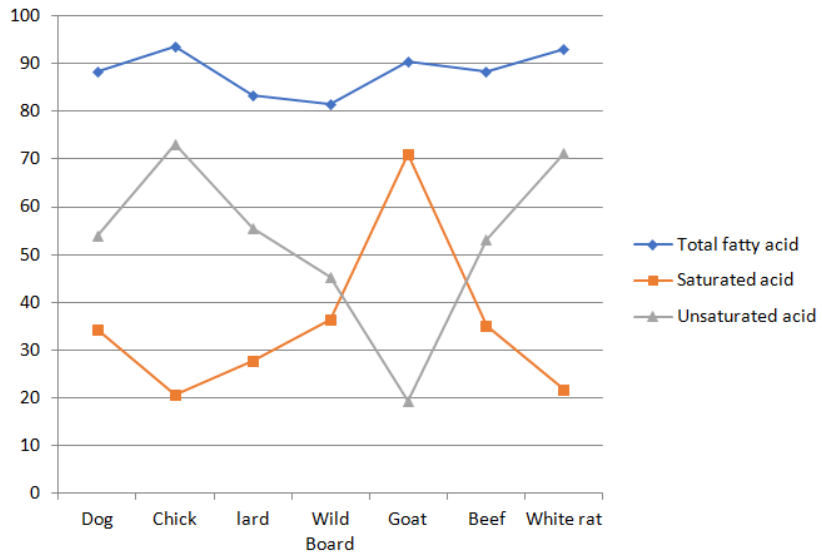

Figure 4 Amount of total fatty acid, saturated acid, and unsaturated fatty acids in the fat of: Wistar rat, dog, wild boar, pork, chick, beef and goat.

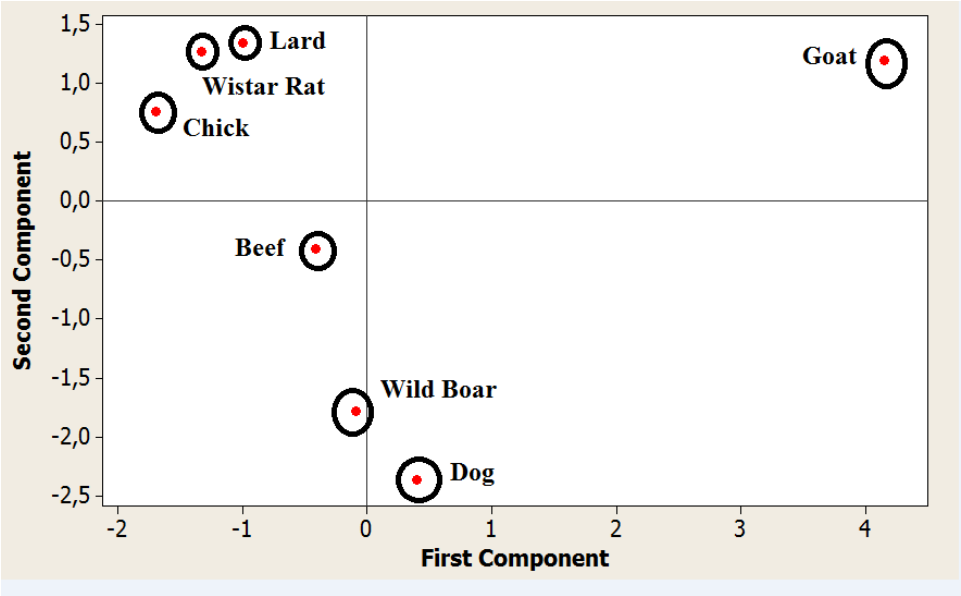

Figure 5 PCA Score Plot of fat from: Wistar rat. dog. chick. pork. wild boar. goat and beef by using fatty acids as a variable.

Table 3 The report of PCA analysis of sausage and some parameters of its Eigen analysis.

Eigenalysis of the Correlation Matrix								
Eigenvalue	3.8699	2.3938	1.0508	0.4382	0.1553	0.0920	-0.0000	-0.0000
Proportion	0.484	0.299	0.131	0.055	0.019	0.011	-0.000	-0.000
Comulative	0.484	0.783	0.914	0.969	0.989	1.000	1.000	1.000
Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Myristate	0.004	0.180	-0.932	0.054	0.237	0.011	0.122	-0.160
Pentadecanoate	0.473	0.219	0.123	0.001	0.003	-0.225	0.802	0.141
Palmitoleate	-0.373	0.346	-0.110	-0.503	-0.490	-0.532	-0.035	0.185
Palmitate	0.470	0.083	-0.145	-0.174	-0.705	0.397	-0.124	-.0224
Margarate	0.036	-0.586	-0.186	0.450	-0.400	-0.507	0.031	-0.028
Linoleate	-0.304	0.432	-0.013	0.634	-0.296	0.262	0.067	0.397
Oleate	-0.267	-0.513	-0.171	-0.328	-0.097	0.418	0.360	0.464
Stearate	0.502	0.003	-0.140	-0.035	0.151	-0.133	-0.437	0.705

Principal Component Analysis of wild boar and other animal fat.

PCA data interpretation is done by reducing data, in which the number of variables in a matrix is reduced to produce new variables while maintaining the information held by the data. The new variables generated are scores or main components **(Rohman and Man, 2012)**. PCA aims to group variables that are correlated with each other and replace them with new groups called main components (principal component) **(Coltro et al., 2005)**. PCA simplifies data by reducing a number of variables to a smaller number of orthogonal variables. This needs a correlation between variables. Although PCA reduces the number of initial variables, PCA retains variability and initial information. PCA also helps provide pattern visualization and correlation analysis **(Miller and Miller, 2010)**. PCA plot scores are presented in Figure 5. The results of replication greatly affect the location of the quadrants obtained, further showing that there are similarities in the physical chemical properties of the fatty acid content. Wistar rat fat is located between chick fat and lard. The results of replication measurements affect the proximity position of the grouping of animal fat.

The results of PCA analysis using Minitab resulted in 8 PCs presented in Figure 6. Each PC displays eigenvalue, proportion, and cumulative values. Eigenvalue variations can explain the data on each PC and show how much influence a variable on the formation of the characteristics of a matrix **(Miller and Miller, 2010).** In Table 3, PC1 with eigenvalue 3.8699 is able to describe 48.4% of the total original data variables while PC2 with eigenvalue 2.3938 is able to describe 21.90% of the total original variables, PC3 with eigenvalue 1.0508 is able to describe 13.10%. Thus, 3 PCs described the illustration data for discriminant analysis of 83.40%.

CONCLUSION

Mass spectroscopy gas chromatography method can be used to authenticate Wistar strain rat with fatty acids content.The content of that fatty acids in white Wistar strain rat is: myristate (0.15 ±0.09)%, palmitoleate (0.73 ±0.54)%, palmitate (19.08 ±3.54)%, linoleic (30.14 ±16.90)%, oleate (40.48 ±2.74)%, and stearic (2.55 ±0.01)%. Chemometrics PCA white rat, dog, wild boar, chick, pork, beef, and goat fat can be grouped.

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OPTIMIZATION OF BREWING TIME AND TEMPERATURE FOR CAFFEINE AND TANNIN LEVELS IN DAMPIT COFFEE LEAF TEA OF ROBUSTA (*COFFEA CANEPHORA*) AND LIBERICA (*COFFEA LIBERICA*)

Dewi Melani Hariyadi, Cynthia Amelia Tedja, Elok Zubaidah, Sudarminto Setyo Yuwono, Kiki Fibrianto

ABSTRACT
Coffee constitutes a plant product high in economic value. Extremely abundant throughout Indonesia, different varieties can be found in each region. Coffee leaves represent a by-product of coffee production which are disposed of without being subjected to further processing. One advantage of coffee leaf waste is that it can be made into coffee leaf tea, to prevent various diseases. The caffeine and tannin content can be optimized by temperature and brewing time. Research on coffee leaf tea remains limited, with little study of Liberica coffee leaf tea. This investigation was to determine the optimal brewing time and temperature of Robusta (Coffea canephora) and Liberica (Coffea liberica) coffee leaf tea. This research used the Response Surface Methodology (RSM) method with Central Composite Design (CCD). Data analysis using RSM method was conducted incorporating two factors. The first factor was the brewing temperature with a minimum value of 91 °C and a maximum value of 99 °C. The second factor was brewing time with minimum and maximum values of three and seven minutes respectively. Identifying the optimal treatment was based on two factors, caffeine and tannin content. Optimized processes were applied to conduct organoleptic tests on 110 untrained panelists using the Rate-All-That-Apply (RATA) method to highlight the majority attributes experienced by the panelists. The optimal brewing temperature and duration for robusta coffee leaf tea were 93.43 °C and 4.80 minutes which produced caffeine and tannin levels of 74.90 mg.100mL^{-1} and 293.01 µg.g^{-1} respectively. In contrast, in the case of Liberica coffee leaf tea, the optimal brewing conditions comprised a temperature of 91.65 °C and duration of 4.84 minutes which produced caffeine and tannin levels of 72.52 mg.100mL^{-1} and 415.87 µg.g^{-1}. Results of sensory analysis showed that the majority produced five significant characteristics, namely: sweet flavor, fire flavor, sweet taste, bitter taste and astringent mouthfeel.

Keywords: caffeine; tannin; Robusta; Liberica; coffee leaf tea

INTRODUCTION
Coffee represents an extremely abundant plant commodity which can potentially be developed into an increasing source of income for Indonesia. The economic value of coffee is relatively high compared to that of other plantation crops. Moreover, its role as a major source of national income is based on its abundance and range of varieties in each region. According to statistics produced by the **General Directorate of Plantations (2016)**, in 2017 plantations in Indonesia occupied an estimated area of 1,227,787 hectares capable of producing 637,539 tons of coffee. The coffee plantations listed in the statistics include those in the community, state and private sectors. Dampit in East Java, at an altitude of 300 – 460m above sea level, is a renowned coffee producing area **(Malang Electronic Data Manager, 2014)**. According to the **Central Statistics Agency of Malang Regency (2018)**, the total coffee production of the Dampit Subdistrict was 2,387 tons from a total cultivated area of 3,373 ha, within which the seedling area covered 6 ha, the productive plant area 2,965 ha and the mature plant area 372 ha. In Indonesia, several varities of coffee have been developed, for example, Arabica, Robusta and Liberica. The two former types contain high levels of active compounds such as quilonic acid, pirogalic acid and, particularly, caffeine **(Ciptaningsih, 2012)**.

For farmers, the only productive part of coffee plants is their beans which can be processed into drinks or food additives. Being considered waste, coffee plant parts such as the leaves are pruned and disgarded. The purpose of pruning is, among other reasons, to improve the quality of coffee, reduce pest attacks and stimulate new growth **(Arief et al., 2011)**. Therefore, rather than discarding the coffee leaves, it is necessary to make further use of them in order to maximise coffee farmer income.

Coffee leaves are as delicious as coffee beans and in an example of product diversification, can substitute for tea leaves in the manufacture of tea drinks. Tea itself is a very

common everyday drink that represents the second most consumed beverage after water **(Damayanthi et al., 2008)**. Tea is made from steeping *Camellia sinensis* leaves which have a distinctive flavor and aroma much appreciated by consumers. However, tea can also be made from other plant parts such as rosella flowers, lemongrass stems, soursop leaves, avocado leaves and coffee leaves. This drink is commonly called herbal tea or tisane **(Somantri and Tanti, 2011)**.

At present, coffee leaf tea is relatively unknown, whereas in Sumatra, coffee leaves are used to produce a drink commonly referred to as "Aia Kawa", believed to contain alkaloids, caffeine, saponins, flavonoids and polyphenols that prevent various diseases **(Pristiana, Susanti and Nurwantoro, 2017)**. The caffeine content in coffee leaf drink is lower than that of coffee beans, rendering it an alternative for producers and individuals who want an affordable soft caffeinated drink. Moreover, tea contains tannin compounds and polyphenol, the latter of which functions as an antioxidant. Elements within coffee leaf tea such as caffeine and tannin are beneficial to the body, but must be optimized in order to maximize their positive effects.

Optimizing the beneficial contents of coffee leaf tea drink such as caffeine and tannin can be achieved based on the temperature and duration of brewing. According to **Schwalfenberg, Genuis and Rodushkin (2013)**, both the beneficial and harmful effects of tea depend on the manner in which it is brewed. The longer the tea is soaked, the more caffeine will be extracted, resulting in oxidation. Several previous studies, such as that conducted by **Retnaningtyas et al. (2016)**, have been limited to the use of Arabica and Robusta coffee leaves, while ones relating to Liberica remain rare. Furthermore, caffeine and tannin are yet to be extensively studied. Therefore, it is necessary to conduct research to optimize caffeine and tannin levels within the parameters of brewing temperature and duration. Moreover, such research should be conducted to determine consumer sensory responses to coffee leaf tea characteristics. Sensory responses are required because caffeine and tannin are compounds that contribute to the flavor of the tea. There would be differences between Robusta and Liberica Coffee leaves tea in terms of caffeine and tannin contents due to different brewing temperature and duration. Thus it may change perceived sensory attributes as well.

This research was aimed to provide information and recommendations to agencies with links to agricultural product development, especially coffee farmers, in order to promote greater use of coffee leaves in tea drinks as part of a food diversification program.

Scientific hypothesis

Temperature and brewing time optimization have an influence on caffeine and tannin levels as well as sensory attributes on Robusta and Liberica coffee leaf tea Dampit.

MATERIAL AND METHODOLOGY

Materials

The raw materials used in this study consisted of Robusta and Liberica coffee leaves obtained form the Dampit region of East Java. The testing materials comprised calcium carbonate, chloroform, distilled water, methanol, folin ciocalteau, Na_2CO_3, tannic acid and caffeic acid. The materials employed for organoleptic testing included mineral water and biscuits as palate cleansers. Dampit region is located in East Java, at an altitude of 300 – 460 m above sea level, is a renowned coffee producing area **(Malang Electronic Data Manager, 2014)**. The period of the harvest is every 4 to 6 months in every year.

Method

The raw materials used in this study were fresh Dampit old Robusta and Liberica coffee leaves. Fresh coffee leaves from older mature leaves were washed and then cut into small pieces. After being reduced in size, the coffee leaves were dried in an oven for three hours at a temperature of 90 °C. The dried coffee leaves were subsequently blended to produce coffee leaf powder. The characteristics of the raw materials analysed in this study were used to determine their chemical content consisting of fresh coffee leaves and coffee leaf powder. The chemical parameters adopted for the analysis of raw materials included water content, caffeine content and tannin content.

The methodology applied in undertaking this study was experimental in character, combining Surface Response Method (RSM) and Rate All That Applied (RATA) data. Application of the RSM method involved an optimization process incorporating the Center Composite design available in the Design Expert DX 9 application (Stat-Ease, Inc.) **(Maharjan et al., 2014; Montgomery, 2001; Montgomery, 2016)**. Chemical analysis related to optimization was conducted based on run provided by the software. The optimization process was conducted with three center points or repetitions to obtain 13 runs or experimental treatments, during which the caffeine and tannin levels were analyzed. The research factors applied were those of temperature and duration of the brewing process. The RSM method produced optimization data subsequently subjected to sensory analysis using the RATA method. A CATA evaluation of the product was conducted to determine the sensory attributes present in coffee leaf tea. As shown from the contents of Table 1, panelists were requested to complete the questionnaire by indicating the attributes they experienced when tasting the sample.

For sensory evaluation, the sensory evaluation based on consumer perception was conducted following RATA (Rate All That Apply) method. This experiment was observed according to Completely Randomized Block Design and the data was further analysed on Minitab 17 (Minitab, LLC, Pty Ltd, Australia) with General Linear Model (GLM) followed by Tukey Post-hoc Test.

The questionnaire results relating to the most prominent sensory attributes were further tested using the RATA method. The panelists were asked to complete the questionnaire by assigning ratings to the strength or dominance of the sensory attributes in the sample.

Data Observation and Analysis

The observations in this study were based on analysis of caffeine, tannin, water content, total acid, pH and color. The coffee leaf tea producing optimal levels of caffeine

and tannin was further analyzed by observing sensory properties using the RATA method.

Caffeine Analysis

Caffeine analysis was completed using the spectrophotometric method **(Maramis, Citraningtyas and Wehantouw, 2013)** which involved adding the sample to hot distilled water, agitating and filtering the resulting liquid prior to adding it to $CaCO_3$ (calcium carbonate). The coffee solution was placed in a separating funnel and extracted four times, with chloroform being added on each occasion. A bottom layer would be formed that could be removed, with the extract (chloroform phase) subsequently being placed in a rotary evaporator until all the chloroform had evaporated. The solvent-free caffeine extract was deposited in a measuring flask, diluted with distilled water and homogenized. The caffeine level was quantified by means of a UV-VIS spectrophotometer at a wavelength of 275 nm. The results having been obtained, a standard curve representing standard caffeine solution as the equation was $y = ax + b$ and resulted correlation coefficient value in each equation.

Tannin Analysis

Tannin analysis was performed using a spectrophotometric method **(de Godoy Pelozo, Carvalho Cardoso and Palazzo de Mello, 2008)**. The sample was extracted using methanol and the levels measured using a folin ciocalteu reagent that formed a complex with tannin producing a dark green blue color. Color intensity was measured using a spectrophotometer at a wavelength of 755 nm. The tannin concentration was quantified by comparing the sample with a standard tanic acid curve.

Sensory Response Analysis using the RATA Method

Sensory response analysis of coffee leaf tea was performed employing the RATA method. Conducting the test involved 110 untrained panelists, aged 18 – 29, drawn from the surrrounding areas of Universitas Brawijaya (Malang). The specified age range was selected because the older the panelists, the less acute their sense of taste **(Mojet, Heidema and Christ-Hazelhof, 2003)**. Sensory testing was performed by completing a questionnaire containing a combination of open and closed questions. The open questions related to the identity of panelists, while the closed questions were designed to elicit their opinions of with regard to the intensity of the perceived attributes of the sample. The resulting data was then recapitulated, processed and analyzed using Minitab to determine the significance of the sensory attributes of the sample. Each rating was scored, followed by the administering of an ANOVA test and a Fischer test.

Statistical analysis

The optimization method used is the Response Surface Method (RSM) method. The RSM methods use a Center Composite Design (CCD) by Design Expert 9 (DX 9) software. Whereas the sensory test uses the Rate-All-That-Apply (RATA) method which is analyzed by the General Linear Model and Fisher's Post-hoc test using the Minitab 17 software.

RESULTS AND DISCUSSION

Characteristics of Raw Materials

Chemical characteristics of raw materials including the analysis of moisture content, caffeine and tannin content are shown in Table 2.

The contents of Table 2 indicated that the water content contained in fresh Robusta coffee leaves was 75.89%, while that of fresh Liberica coffee leaves was 72.93%. According to **Angga et al. (2018)**, the water content of old tea leaves was approximately 70%. Moreover, research conducted by **Kristiningrum, Cahyanti and Wulandari** in **2016** into Jember Arabica and Robusta coffee leaves indicated that old coffee leaves had a moisture content of 75.79 – 82.82%. The moisture content of Robusta coffee leaf powder was 6.84%, while that of Liberica was 5.5%. The criterion of good tea quality is a maximum moisture content of 10% **(Čížková et al., 2008)**. The drying process can reduce the moisture content of fresh coffee leaves by approximately 60 – 70% **(Deb and Pou, 2016)**.

The caffeine level in fresh coffee leaves was higher than the level of caffeine in dried coffee leaf powder. The caffeine content of fresh robusta leaves was 0.63%, while that of the powder was 0.23%. The caffeine content of fresh Liberica leaves was 0.43% and the powdered caffeine level was 0.36%. According to the literature, the caffeine content of coffee leaves is relatively low compared to that of coffee beans which is 1.6 – 2.4% **(Khotimah, 2014)**. The amount of caffeine in coffee leaves can vary and is influenced by the cultivation area, growth conditions, environment, season, leaf age and specific production system **(Heckman, Weil and Gonzales de Mejia, 2010)**.

The tannin content of fresh coffee leaves was higher than the that of dried coffee leaf powder. The tannin content of fresh robusta leaves was 19.24%, while that of the leaf powder was 12.27%. The tannin content of fresh liberica leaves was 18.38%, while that of leaf powder was 13.83%. The literature on the subject states that tea leaves contain numerous tannin compounds amounting to 13.76%. These affect both astringence and bitter taste, but both decrease after processing **(Karori et al., 2007)**.

Optimization of Caffeine and Tannin Content of Robusta Coffee Leaf Tea

Optimization of Robusta coffee leaf tea was conducted using the variables of brewing temperature and brewing time. This approach was in accordance with the experimental design of Design Expert 9.0 using Response Surface Methodology (RSM). The observed responses consisted of the caffeine and tannin levels.

The results of the study of the relationship between brewing temperature and duration and the levels of caffeine and tannin are contained in Table 3.

Analysis of the 13 treatment combinations indicated that the response value of caffeine content tended to be low, namely; 67.85 $mg.100mL^{-1}$, when produced by a brewing process at a temperature of 89.34 °C and of five minutes' duration. The response value of the caffeine level tended to be high at 83.03 $mg.100mL^{-1}$ which occured in the

brewing process at a temperature of 99 °C and duration of seven minutes. According to the research conducted by **Putri and Ulfin (2015)**, caffeine content is influenced by the extraction conditions, namely; temperature and extraction duration. The highest caffeine level is found in the temperature of 100 °C. In addition, a longer extraction time can increase the caffeine level in tea. Caffeine is a compound that dissolves readily in hot water. At a temperature of 25 °C its solubility is 2.17 g.100mL^{-1}, while at 80 °C the solubility is 18 g.100mL^{-1}, and at 100 °C, the solubility is 67 g.100mL^{-1}**(Mumin et al., 2006)**.

The tannin level with a high tendency value of 314.857 µg.g^{-1} resulted from a brewing process at a temperature of 95 °C and of five minutes' duration. Meanwhile, tannin content with a value as low as 225.77 µg.g^{-1} was obtained at a brewing temperature of 89.33 °C and a brewing time of five minutes. According to **de Hoyos-Martínez et al. (2019)**, all forms of tannin dissolve in water, methanol, ethanol and acetone, demonstrating a high level of solubility that increases when dissolved in hot water. Tannin will break down into pyrogallol, pyrocatechol and phloroglucinol when heated to temperatures of 2,100 F – 2,150 F (98.89 °C – 101.67 °C).

Statistical analysis has demonstrated that increased caffeine content is affected by both the brewing temperature and brewing time. These factors have a significant effect on caffeine content where the higher the linear temperature, the higher the resulting level of caffeine.

Moreover, the higher the water temperature in the brewing process, the greater the ability of water to extract the chemical content of tea and the longer the brewing time. The latter will affect the level of dissolved ingredients, color intensity and aroma. Increasing the brewing time will lengthen the contact time between the hot water and tea, thereby rendering the extraction process more effective **(Saklar et al., 2015)**.

Optimization of Brewing Temperature and Time of Robusta Coffee Leaf Tea and Desired Responses

Solution for optimal process based on Design Expert 9.0 application was obtained by determining the desired variable and response criteria and can be seen in Table 4.

In Table 4, the variable criteria for brewing temperature and brewing time chosen were within a specific range. This was because it was expected that the brewing process would be carried out at a temperature and duration between the specified upper and lower limits. In the caffeine content response variable, the selected criteria chosen was that of minimize, because the desired caffeine content was the lowest, thereby rendering it safe for consumption by individuals sensitive to caffeine.

In contrast, the response of the desired tannin content was maximum because to maximize its functional properties such as astringent and antibacterial activity. Based on these criteria, the Design Expert program provides an optimization solution that can be seen in Table 5.

The data in Table 5 indicates that the solution provided by the program had a desirability level of 0.64 or a level of accuracy of the predicted value with an optimization value of 0.64. The desirability value was indicated by the value of 0 – 1, where the higher the value indicated the more suitable the combination of process parameters obtained to achieve the optimal combination with the desired response variable **(Melati, 2012)**. The recommended optimal processing point in terms of brewing temperature was 93.43 °C and of cooking time was 4.80 minutes. The predicted caffeine content following the brewing process was 74.90 mg.100mL^{-1}, while that of tannin was 293.01 µg.g^{-1}.

Optimization of Liberica Coffee Leaf Tea Caffeine and Tanin Content

Optimization of Liberica coffee leaf tea was carried out using variables in the form of brewing temperature and time and in accordance with the experimental design by Design Expert 9.0 using Response Surface Methodology (RSM). The observed responses were caffeine and tannin content. The results of the study of the relationship between brewing temperature and brewing time and caffeine and tannin content can be seen in Table 6.

The optimized caffeine content response represented the amount of caffeine contained in 100 mL of steeped Liberica coffee leaf tea. Based on the analysis of 13 treatment combinations, the response value of caffeine content, which tended to be low at 61.68 mg.100mL^{-1}, was produced by brewing for three minutes at a temperature of 91 °C. The response value of caffeine content which tended to be high at 91.83 mg.100mL^{-1} resulted from brewing at a temperature of 100.66 °C for five minutes.

The result showed that the highest caffeine content was found following the longest time again range at 100 °C.

The tannin content which had a high tendency value was 464.99 µg.g^{-1} obtained during a brewing process at temperature of 95 °C and of five minutes' duration. Tannin content tended to have a value as low as 280.57 µg.g^{-1} obtained at a brewing temperature of 99 °C and a brewing time of three minutes.

Statistical analysis indicated that brewing temperature and time exert a significant influence on caffeine content.

Optimization of Temperature and Time of Liberica Coffee Leaf Tea and Desired Response

The solution for optimal processes based on Design Expert 9.0 application is obtained by determining the desired variable and response criteria as shown in Table 7. In Table 7, the variable criteria for the brewing temperature and brewing time chosen were in a range. This was due to the expectation that the brewing process would be carried out at a temperature and duration between the specified upper and lower limits. In the caffeine content response variable, the criteria chosen was 'minimize', because the desired caffeine content was the lowest. Therefore, it was safe for consumption by those sensitive to caffeine. In contrast, the response to the desired tannin content was 'maximum', due to the optimizing of its functional properties. Based on these criteria, the Design Expert program produced an optimization solution that is contained in Table 8. The solution provided by the program quantified desirability as 0.69. In other words, the level of accuracy of the predicted optimization value was one of 0.69.

Table 1 Sensory Attributes.

Attributes		Description
Odor	Green	Resembling leaves, vegetables or herbal plants
	Floral	Reminiscent of jasmine and rose petals
	Spicy	Possessing the aroma of cloves, pepper or ginger
	Fruity	Similar to the odor of apples, melons, berries
	Marine	Resembling seaweed
	Gouda	Nuts, for example almonds and peanuts
	Sweet	Sweet like honey, sugar or caramel
	Fire	Burning like ash or smoke
	Minerals	Reminiscent of minerals such as metal or chalk
	Earth	Resembling the odor of soil, moss or compost
	Wood	Reminiscent of timber such as pine or oak
Taste	Sweet	Sweet taste
	Sour	Sour taste
	Bitter	Bitter taste
Flavor	Green	Resembling leaves, vegetables or herbal plants
	Floral	Similar to the flavor of jasmine and rose flowers
	Spicy	Spices such as clove, pepper, or ginger
	Fruity	Fruits like apples, melons, berries
	Marine	Resembling sea angina or seaweed
	Gouda	Nuts like almonds, peanuts
	Sweet	Sweet like honey, sugar, or caramel
	Fire	Burnt flavors such as ash or smoke
	Minerals	Resembling minerals like metal or chalk
	Earth	Resembling soil, moss, or compost
	Wood	Resembling timber such as pine, oak
Mouthfeel	Astringent	Dry and slightly viscous sensation in the mouth
	Oily	Oily sensation in the mouth

Table 2 Raw Material Analysis Data.

Parameters	Robusta		Liberica	
	Leaf*	Powder*	Leaf	Powder
Moisture Content (%)	75.89	6.84	72.93	5.5
Caffeine Content (%)	0.63	0.23	0.43	0.36
Tannin Content (%)	19.24	12.27	18.38	13.83

Table 3 Research Data on Robusta Coffee Leaf Tea.

Std.	"Run"	Factor 1	Factor 2	Response 1	Response 2
		A:Brewing Temperature	B:Brewing Duration	Caffeine Content	Tannin Content
		°C	minute	$mg.100mL^{-1}$	$\mu g.g^{-1}$
13	1	95	5	76.55	290.70
8	2	95	7.83	78.68	285.77
1	3	91	3	74.2	231.22
5	4	89.34	5	67.85	225.77
6	5	100.66	5	81.1	254.34
9	6	95	5	76.98	297.71
2	7	99	3	80.2	241.61
3	8	91	7	75.7	265.51
12	9	95	5	76.93	314.86
7	10	95	2.17	70.93	262.13
4	11	99	7	83.03	281.61
10	12	95	5	77.15	305.51
11	13	95	5	77.3	304.21
13	1	95	5	76.55	290.70

Table 4 Criteria for Variables and Desired Responses.

Name	Goal	Limit	Limit	Importance
Brewing Temperature	within range	91	99	3
Brewing Time	within range	3	7	3
Caffeine Content	minimize	67.85	83.03	3
Tannin content	maximize	225.77	314.86	3

Table 5 Robusta Coffee Leaf Tea Optimal Temperature Point and Brewing Time.

No.	Brewing Temperature	Brewing Time	Caffeine Content	Tannin Content	Desirability	
1	93.43	4.80	74.90	293.01	0.64	Selected

Table 6 Research Data on Liberica Coffee Leaf Tea.

Std.	"R"	Factor 1	Factor 2	Response 1	Response 2
		A:Brewing Temperature	B:Brewing Duration	Caffeine Content	Tannin Content
		°C	minute	mg.100mL^{-1}	µg.g^{-1}
13	1	95	5	80.08	446.29
8	2	95	7.83	83.5	388.36
1	3	91	3	61.68	320.57
5	4	89.34	5	71.98	376.68
6	5	100.66	5	91.83	320.57
9	6	95	5	81.08	399.01
2	7	99	3	86.55	280.57
3	8	91	7	75.18	392.52
12	9	95	5	79.95	398.23
7	10	95	2.17	76.65	290.44
4	11	99	7	80.31	345.25
10	12	95	5	79.45	455.64
11	13	95	5	80.23	464.99
13	1	95	5	80.08	446.29

Table 7 Criteria for Variables and Desired Responses.

Name	Goal	Limit	Limit	Importance
A:Brewing Temperature	is in range	91	99	3
B: Brewing Time	is in range	3	7	3
Caffeine Content	minimize	61.68	91.83	3
Tannin Content	maximize	280.57	464.99	3

Table 8 Liberica Coffee Leaf Tea Optimal Temperature Point and Brewing Time.

No.	Brewing Temperature	Brewing Time	Caffeine Content	Tannin Content	Desirability	
1	91.65	4.84	72.52	415.87	0.69	Selected

Desirability value was indicated by a value between 0 and 1, where the higher the value the greater the suitability of the combination of process parameters obtained to achieving the optimal combination with the desired response variable **(Melati, 2012)**. The recommended optimal processing point was 91.65 °C for brewing temperature and 4.84 minutes for cooking time. The prediction result of caffeine content obtained from the brewing process was equal to 72.52 mg.100mL^{-1} with a tannin content of 415.87 µg.g^{-1}.

Sensory Characterization of Coffee Leaf Tea

Panelist Profiles

The panelists involved in testing the sensory characteristics of coffee leaf tea were untrained individuals of average intelligence who had not been formally trained, but were capable of differentiating and communicating the reactions resulting from orgnoleptic assessment **(Ayustaningwarno, 2014)**. The 110 participating panelists were composed of 24 males and 86 females as shown in Table 9.

The age range of panelists in this study was one of 18 – 29 years. Based on research conducted by **Hanspal (2010)**, tea is most popular among consumers within the 18 to 36 years age range. Significantly, the declining sense of taste of panelists will compromise their ability to differentiate between flavours from the age of 45 **(Choi, 2019)**.

Sensory Attribute Characterization of Robusta and Liberica Coffee Leaf Teas Using the RATA (Rate-All-That-Apply) Method

The measuring of sensory attributes used the RATA sensory evaluation method (Rate-All-That-Apply). This represents a sensory evaluation method utilizing a questionnaire instrument in which there are intensities for each sensor attribute. The RATA method requires panelists to select the intensity of sensory attributes of the product. Response boxes and can be left blank if no attributes are detected in the product **(Ares et al., 2014)**. The intensity used to describe the sensory attributes in the questionnaire ranged from numbers 1 – 5. Attribute number 1 represents 'Very lacking', number 2 'Lacking', number 3 'Moderate', number 4 'High' and number 5 'Very high'. After completion of the questionnaire, panelist responses to coffee leaf tea were presented in a spider chart contained in Figure 1. The responses represented the average intensity of the sensory attributes from 110 panelists.

Table 9 RATA Sensory Panelist Profile Analysis of Coffee Leaf Tea.

No.	Item	Item Options	Number of People
1.	Sex	a. Male (M)	24
		b. Female (F)	86
2.	Age	a. 18 – 21 (Late teen)	53
		b. 22 – 29 (Early adulthood)	57

Table 10 Sensory Attributes of Coffee Leaf Tea Resulting from Different Leaf Types.

Attributes	*p*-value (Coffee Leaf Type)
Sweet Flavor	0.005
Fire Flavor	0.001
Sweet taste	0.000
Bitter Taste	0.000

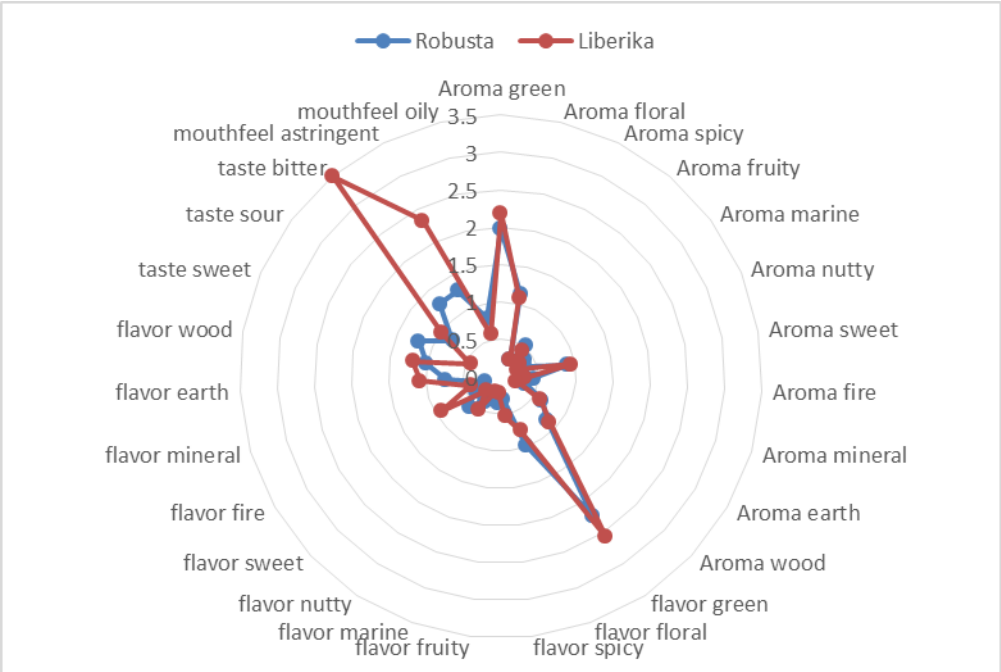

Figure 1 Average Response of the Intensity of Robusta and Liberica Coffee Leaf Tea Sensory Attributes.

Panelist Responses to Coffee Leaf Tea Sensory Attributes to Leaf Types

This study used samples of Robusta and Liberica coffee leaves brewed in accordance with the optimal brewing process solution obtained from the Design Expert application. Robusta coffee leaves were brewed at 93.43 °C for 4.80 minutes. In contrast, the Liberica coffee leaves were brewed at a temperature of 91.65 °C for 4.84 minutes. After sensory evaluation, the data was processed using ANOVA test on Minitab 17 software. The results of the *p*-value analysis of sensory attributes that differed significantly according to the specific variety of coffee leaves can be seen in Table 10.

Data analysis of *p*-value variance for the sensory attributes of the different types of Robusta and Liberica coffee leaves confirmed the existence of five significantly different sensory attributes (p-value <0.05), namely: sweet flavor, fire flavor, sweet taste, bitter taste and astringent. The results were significantly different indicating that the panelists experienced a difference in the intensity of the sensory attributes of coffee leaf tea present in the different types of coffee leaves. The analysis results were subsequently subjected to Fisher tests of significantly different sensory attributes.

Sweet Flavor

Sweet flavor is a flavor often associated with the impression of sweetness in items such as fruits and flowers. Sweet Flavor is also considered to be contained in sugar, honey, or caramel **(Lee and Chambers, 2007)**. The ANOVA test result indicated that the variance in sweetness in the different types of coffee leaves differed significantly. It can be concluded that the sweet flavor attribute will become stronger when approaching number 5 and weaker when approaching number 0. The different notation in each sample shows them to be significantly different. Sweet flavor in steeped tea leaves can be contributed to by simple sugars contained in coffee leaves such as glucose, sucrose, fructose, and various volatile compounds such as linalool, dihydroactinidiolide, coumarin, and phytol **(Lee, Chambers and Chambers, 2013)**. Compounds that also contribute to sweet flavor, include: nerol, phenylacetaldehyde, 5-octanolide, menalool oxide and geraniol **(Yashin et al., 2015)**. Liberica coffee leaves had a sweeter flavor because it contained more glucose, sucrose, fructose, and various volatile compounds than Robusta leaves.

Fire Flavor

Fire flavor is associated with the aroma of soil, ash, roasted pepper, coffee and smoke, in addition to heat in the throat **(Kim, Lee and Kim, 2016)**. ANOVA analysis of fire flavor response in the various types of coffee leaves indicated significant differences. It can be concluded that the fire flavor attribute strengthens when approaching number 5 and weakens when approaching 0. The different notation in each sample shows it to be significantly different. Fire flavor in steeped tea leaves can be contributed to by 1-ethyl-1H-pyrrole-2-carboxaldehyde, 3-ethyl-4-methyl-1H-pyrrole-2,5-dione, 3-ethyl-3-methyl-2,5- pyrrolidinedione, coumarone, and coumarine **(Yashin et al., 2015)**.

In addition, research undertaken by **Angga et al. (2018)** found five aromatic compounds burning in coffee leaves, namely: 2-furanmethanol, safranal, benzeneethanol (CAS), guaiacol and 3,5-cocoa pyrazine.

Liberica coffee leaves had a more fire flavor because it contained more safranal, guaiacol, coumarone and coumarine than Robusta leaves.

Sweet taste

Sweet taste is caused by aliphatic organic compounds containing hydroxy groups (OH), several amino acids, aldehydes and glycerol which are commonly found in food containing simple carbohydrates **(Diez-Simon, Mumm and Hall, 2019)**.

The results of ANOVA analysis of sweet taste response to the differences in the types of coffee leaves varied significantly. It can be concluded that the sweet taste attribute will become stronger when approaching number 5 and weaker when approaching number 0. The contrasting notation in each sample shows it to be significantly different. Sweet taste is often associated with aldehydes and ketones which contain carbonyl groups **(Chang, Waters and Liman, 2010)**. According to research conducted by **Angga et al. (2018)**, coffee leaves contain 15 aldehyde group compounds, six alcohol group compounds and four ketone group compounds. Furthermore, sweet taste is also considered a pleasant sensation produced by the presence of sugar and several other substances. Liberica coffee leaves had a sweeter taste because it contained more aldehyde and ketone than Robusta leaves.

Bitter Taste

Bitter taste is caused by the presence of phenol compounds, flavonoids, isoflavones, terpenes and glucosinolates which exude a bitter, sharp or astringent odor **(Drewnowski and Gomez-Cameron, 2000)**. The results of ANOVA analysis of bitter taste response to the differences in the types of coffee leaves varied significantly. It can be concluded that the bitter taste attribute will strengthen when approaching number 5 and weaken when approaching number 0. The contrasting notation in each sample showed that it was significantly different. Liberica coffee leaves had a much more bitter taste in the ratings because it contained more flavonoid and tannin than Robusta leaves.

Compounds that contribute to bitter taste in tea are phenolic compounds such as flavonoids (quercetin), flavans (catechins, epicatechin and epicatechin gallate, epigalocatechingallate). High levels of flavonoid and tannin in tea contribute to an increase in the bitter taste of tea **(Mahmood, Akhtar and Khan, 2010)**.

Astringent Mouthfeel

Mouthfeel is a physical sensation in the oral cavity caused by food or drink but which differs from taste **(Mouritsen and Styrbæk, 2017)**. Astringency constitutes dryness in the mouth and a slight sensation of stickiness caused by the presence of astringent substances in plants (in the form of leaves, flowers and fruit) that are not yet ripe **(Laaksonen, 2011)**. **Rossetti et al. (2009)** state that astringent substances are able to constrict body tissue where the lubricant is produced and disappears due to the

deposition of proteins contained in the saliva that lines and lubricates the oral cavity. The result of ANOVA analysis of astringent response to variations in the types of coffee leaves was significantly different. It can be concluded that the astringent attribute will get stronger when approaching number 5 and weaker when approaching number 0. Varying notation in each sample shows that the sample is significantly different. Liberica coffee leaves had a much more astringent mouthfeel because it contained more caffeine, tannin and catechin than Robusta leaves. Astringence is important in determining the sensory quality of drinks such as tea, coffee, juice or wine. The compounds that play the most dominant role in influencing astringence are tannin and catechin. Moreover, astringence is also caused by caffeine and flavonol glycosides **(Zhang et al., 2018)**. In addition to five significantly different attributes, 22 sensory attributes exist that are not significantly different from the various types of coffee leaves.

CONCLUSION

The temperature and length of time required to optimize caffeine and tannin levels in Robusta coffee leaf tea are 93.43 °C and 4.80 minutes respectively. The caffeine level of Robusta coffee leaf tea with optimal steeping amounted to 74.90 mg.100mL^{-1} and Robusta coffee leaf tea tannin level with optimal steeping amounted to 293.01 µg.g^{-1}. On the other hand, the brewing temperature and time required to optimize the levels of caffeine and tannin in Liberica coffee leaf tea are 91.65 °C and 4.84 minutes respectively. The caffeine level in Liberica coffee leaf tea with optimal steeping is 72.52 mg.100mL^{-1}, while the level of Liberica coffee leaf tea tannin with optimal steeping is 415.87 µg.g^{-1}. Sensory responses of consumers towards Robusta and Liberica coffee leaf tea that have been optimized for caffeine and tannin levels indicate five significant attributes, which are sweet flavor, fire flavor, sweet taste, bitter taste and astringent mouthfeel.

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THE CHARACTERISTIC OF SHEEP CHEESE "BRYNDZA" FROM DIFFERENT REGIONS OF SLOVAKIA BASED ON MICROBIOLOGICAL QUALITY

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ABSTRACT

The aim of our study was to describe microorganisms which occur in the traditional Slovak cheese „Bryndza". There were a total of 60 cheese samples collected from ten different farms during May 2019. The microbiota studies included the total bacterial count, coliforms, enterococci, lactic acid bacteria, yeasts and microscopic fungi. The total bacterial counts were cultivated on plate count agar at 30 °C in aerobic conditions, lactic acid bacteria on MRS at 37 °C in anaerobic conditions, coliform on VRBL and VRBG at 37 °C in aerobic condition, yeasts and microscopic fungi on MEA at 25 °C under aerobic condition. Gram-positive, Gram-negative and yeasts isolates were identified with MALDI-TOF MS Biotyper. Totally, a number of 1175 isolates of G^-, G^+ and yeast were identified with score higher than 2 and moulds. *Escherichia coli* and *Stenotrophomonas maltophilia* were the most frequently identified species of Gram-negative and *Leuconostoc mesenteroides ssp. mesenteroides* and *Lactococcus lactis ssp. lactis* from Gram-positive bacteria. *Yarrowia lipolitica* and *Kluyveromyces lactis* were the most distributed yeasts. Lactic acid bacteria group was represented by *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus.* The most abundant genera of lactic acid bacteria were *Lactobacillus* with 11 species. This study describes the indigenous microbiota of the traditional ewe's milk cheeses from Slovakia.

Keywords: isolation and identification of microorganisms; MALDI TOF MS Biotyper; Slovak ewe's cheese

INTRODUCTION

Slovak „Bryndza" is a natural white, gently spreadable, slightly moist fresh ripened cheese with curds and own texture, made in a traditional way from well-fermented ripened ewe's lump cheese.

A characteristic feature of the production of Slovak „Bryndza" is the crushing and grinding of mature ewe's or a mixture of ewe's and cow's lump cheese and their mixing with salt or specially prepared saline solution to achieve the required composition, which distinguishes this production from the production of other ewe's cheese produced outside Slovakia. Its characteristic sensory attributes are due to the natural microflora contained in raw ewe's milk and ewe's lump cheese and to the characteristic production method. The basic raw material for the production of Slovak „Bryndza" is ewe's lump cheese or a mixture of ewe's and cow's lump cheese, or a mixture of cured ewe's lump cheese and cow's lump cheese aged under specific conditions **(Commission Regulation No. 676/2008)**.

Microorganisms represent without doubt the largest group of living organisms in the world, with only a small fraction of microbial species which have been identified until now. They can be highly diverse in their biochemistry, physiology and nutritional modes. Most of them are reproducing swiftly and the significant plasticity of their genome allows them to easily adapt to changing environmental conditions, as well as perform a variety of essential ecosystem functions, on which food production depends on. According to **FAO (2009)**, the main functional groups for food processing are beneficial microorganisms (fermentation and probiotics). Microbial food cultures include bacterial food cultures, fungi and yeasts. These microorganisms determine the characteristics of the fermented food, e.g., acidity, flavour and texture, as well as health benefits that go beyond elementar nutrition **(Vogel et al., 2011)**.

The aim of our study was to isolate and identificate the microorganisms from Slovak ewe's cheese „Bryndza" obtained from different Slovak regions.

Scientific hypothesis

Slovak „Bryndza" is specific traditional food product with various microorganisms, which has positive and negative role of quality.

Hypothesis no. 1: There are a lot of different bacteria and yeast species presented in the traditional Slovak sheep cheese called „Bryndza".

Hypothesis no. 2: There are microscopic filamentous fungi presented in the traditional Slovak sheep cheese „Bryndza".

MATERIAL AND METHODOLOGY

There were 60 samples of Slovak ewe's cheese „Bryndza“ from east, middle and west part of Slovakia evaluated for microbiological quality in our study. All samples were obtained in May 2019. These samples were placed in sterile sample containers and transported on ice to the laboratory for microbiological investigations. Samples were kept in a refrigerator (4 ±1 °C) until the testing began. The primary dilution of the ewe's cheese was made for preparing the samples for testing: a 5 mL of sample material was added to 45 mL of 0.87 % sterile saline. Then the serial dilutions (10^{-2} to 10^{-4}) were done and a 100 μL of each dilution was plated out.

Determination of total bacterial count

Plate count agar (PCA, Sigma-Aldrich®, St. Louis, USA) for total microbial count enumeration was used. Inoculated plates were incubated at 30 °C for 24 – 48 h and then examined for the characteristics of bacterial colonies.

Isolation of coliform bacteria

The Violet red bile lactose agar (VRBGA, Sigma-Aldrich®, St. Louis, USA) for enumeration of coliforms bacteria was used. Inoculated plates were incubated at 37 °C for 24 – 48 h and then examined for the characteristics of typical colonies.

Isolation of enterococci

Enterococcus selective agar (ESA, Sigma-Aldrich®, St. Louis, USA) for enumeration of enterococci was used. Inoculated plates were incubated at 37 °C for 24 – 48 h and then examined for the characteristics of typical colonies.

Isolation of Lactic Acid Bacteria (LAB)

MRS (Main Rogose agar, Oxoid, UK), MSE (Mayeux, Sandine and Elliker in 1962, Oxoid, UK), and APT (All Purpose TWEEN® agar, Oxoid, UK) agars were used for enumeration of LAB including lactobacilli, leuconostocs and lactic acid streptococci as well as other microorganisms with high requirements for thiamine (Sigma-Aldrich®, St. Louis, USA). Inoculated agars were incubated at 30 °C for 72 h anaerobically and then the bacterial growth was evaluated.

Isolation of yeasts

Malt extract agar (Sigma-Aldrich®, St. Louis, USA) and acid base indicator bromocresol green (Sigma-Aldrich®, St. Louis, USA) (0.020 $g.L^{-1}$) were used for yeasts identification. Inoculated plates were incubated at 25 °C for 5 days aerobically and then the growth was evaluated.

Sample preparation and MALDI-TOF MS measurement

Prior to the identification, the bacterial and yeasts colonies were subcultured on TSA agar (Tryptone Soya Agar, Oxoid, UK) at 37 °C for 18 – 24 h. One colony of eight bacterial isolate was selected. Subsequently, the identification was performed using the Maldi TOF MS Biotyper as was described by **Kačániová et al. (2019)**. We identified totally 870 isolates with a score higher than 2 **(Kačániová et al., 2019)**.

Identification of microscopic fungi

Microscopic fungi were identified to species level according to the manuals of **Samson et al. (2002)**, **Samson and Frisvad (2004)**, **Pitt and Hocking (2009)**.

Statistical analysis

All experiments were carried out in triplicate and the results reported are the results of those replicate determinations with standard deviations.

RESULTS AND DISCUSSION

Different groups of microorganisms were isolated from the 60 ewe's cheese „Bryndza“ samples (Table 1). Total bacterial count in ewe's cheese ranged from 3.87 ±0.58 $CFU.g^{-1}$ from west Slovak producers to 4.32 ±0.17 $CFU.g^{-1}$ from middle Slovak producers. Generally, the coliform bacteria ranged from 3.46 ±0.26 $CFU.g^{-1}$ from east Slovak producers to 3.64 ±0.19 $CFU.g^{-1}$ for bryndza from middle Slovak producers. The number of lactic acid bacteria ranged from 3.14 ±0.09 $CFU.g^{-1}$ from east Slovak producers to 3.24 ±0.21 $CFU.g^{-1}$ in the bryndza cheeses from west Slovak producers. Table 2a and Table 2b showes isolated species of bacteria.

The eukaryotic microorganisms were represented largely by members of the genera of *Dipodascus* and *Kluyveromyces*, which were present at a level of 99 isolates and 60 isolates, and by other yeasts, which were present in *Candida* genera (Figure 3). The moulds were present generally at middle levels, with the most colonies of *Rhizopus* spp. With 21 isolates. All samples contained high numbers of lactic acid bacteria belonging to genera *Lactobacillus, Lactococcus* and *Leuconostoc*. In order to obtain a better view of the lactic acid bacteria isolates, the *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc and Pediococcus* strains were identified by mass spectrometry assays. Representatives of the species *Leuconostoc mesenteroides* ssp. *mesenteroides* were isolated and identified in all bryndza cheese samples (Figure 2).

Different *Lactobacillus* species, such as *Lb. brevis*, *Lb. delbrueckii*, *Lb. fermentum, Lb. helveticus, Lb. harbinensis, Lb. jonsonii, Lb. paracasei* ssp. *paracasei, Lb. plantarum, Lb. paraplantarum, Lb. rhamnosus* and *Lb. suebicus*, were identified in the bryndza cheese samples from all producers (Figure 2). The most isolated species were *Lb. brevis, Lb. fermentum* and *Lb. plantarum.*

There were totally 1175 isolates identified by mass spectrometry include G^-, G^+ and microscopic filamentous fungi in our study. Together 199 isolates were (Figure 1) isolated and identified from G^- and most frequently species was *Escherichia coli*, 599 isolates from G^+ with most isolated species *Leuconostoc mesenteroides* ssp. *mesenteroides* (Figure 2) and 377 isolates of yeast and molds where the most frequently isolated species was *Yarrowia lipolitica* (Figure 3).

Table 1 Groups of microorganisms in ewe's cheese „Bryndza".

Microorganisms	Content CFU.g^{-1}		
	east	middle	west
Total bacterial count	4.05 ±0.45	4.32 ±0.17	3.87 ±0.58
Coliforms bacteria	3.64 ±0.19	3.60 ±0.21	3.46 ±0.26
Enterococci	2.77 ±0.23	2.71 ±0.17	2.67 ±0.29
Lactic acid bacteria	3.24 ±0.21	3.17 ±0.10	3.14 ±0.09
Yeats and molds	2.41 ±0.19	2.28 ±0.14	2.18 ±0.10

Table 2a Isolated family, genera and species from ewe's cheese.

Family	Genera	Species
Moraxellaceae	*Acinetobacter*	*Acinetobacter baumannii*
Moraxellaceae	*Acinetobacter*	*Acinetobacter tandoii*
Bacillaceae	*Bacillus*	*Bacillus pumilus*
Saccharomycetaceae	*Candida*	*Candida catenulate*
Saccharomycetaceae	*Candida*	*Candida krusei*
Saccharomycetaceae	*Candida*	*Candida lusitaniae*
Saccharomycetaceae	*Candida*	*Candida rugose*
Saccharomycetaceae	*Candida*	*Candida utilis*
Enterobacteriaceae	*Citrobacter*	*Citrobacter braakii*
Enterobacteriaceae	*Citrobacter*	*Citrobacter koseri*
Davidiellaceae	*Cladosporium*	*Cladosporium* spp.
Dipodascaceae	*Dipodascus*	*Dipodascus candidum*
Dipodascaceae	*Dipodascus*	*Dipodascus silvicola*
Enterobacteriaceae	*Enterobacter*	*Enterobacter cloacae*
Enterobacteriaceae	*Enterobacter*	*Enterobacter ludwigii*
Enterococcaceae	*Enterococcus*	*Enterococcus faecalis*
Enterococcaceae	*Enterococcus*	*Enterococcus faecium*
Enterococcaceae	*Enterococcus*	*Enterococcus hirae*
Enterobacteriaceae	*Escherichia*	*Escherichia coli*
Enterobacteriaceae	*Hafnia*	*Hafnia alvei*
Enterobacteriaceae	*Klebsiella*	*Klebsiella oxytoca*
Enterobacteriaceae	*Klebsiella*	*Klebsiella pneumoniae* ssp. *ozaenae*
Enterobacteriaceae	*Klebsiella*	*Klebsiella pneumoniae* ssp. *pneumonia*
Saccharomycetaceae	*Kluyveromyces*	*Kluyveromyces lactis*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus brevis*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus delbrueckii*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus fermentum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus helveticus*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus harbinensis*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus johnsonii*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus paracasei* ssp. *paracasei*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus plantarum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus paraplantarum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus rhamnosus*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus suebicus*
Streptococcaceae	*Lactococcus*	*Lactococcus lactis* ssp. *lactis*
Streptococcaceae	*Lactococcus*	*Lactococcus lactis* ssp. *cremoris*
Lactobacillaceae	*Leuconostoc*	*Leuconostoc mesenteroides* ssp. *mesenteroides*

Table 2b Isolated family, genera and species from ewe's cheese.

Family	Genera	Species
Microbacteriaceae	*Microbacterium*	*Microbacterium liquefaciens*
Lactobacillaceae	*Pediococcus*	*Pediococcus acidilactici*
Saccharomycetaceae	*Pichia*	*Pichia cactophila*
Saccharomycetaceae	*Pichia*	*Pichia fermentas*
Enterobacteriaceae	*Raoultella*	*Raoultella ornithinolytica*
Mucoraceae	*Rhizopus*	*Rhizopus* spp.
Enterobacteriaceae	*Serratia*	*Serratia liquefaciens*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus aureus* ssp. *aureus*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus pasteuri*
Xanthomonadaceae	*Stenotrophomonas*	*Stenotrophomonas maltophilia*
Dipodascaceae	*Yarrowia*	*Yarrowia lipolytica*

Figure 1 Gram negative bacteria isolated from ewe's cheese bryndza.

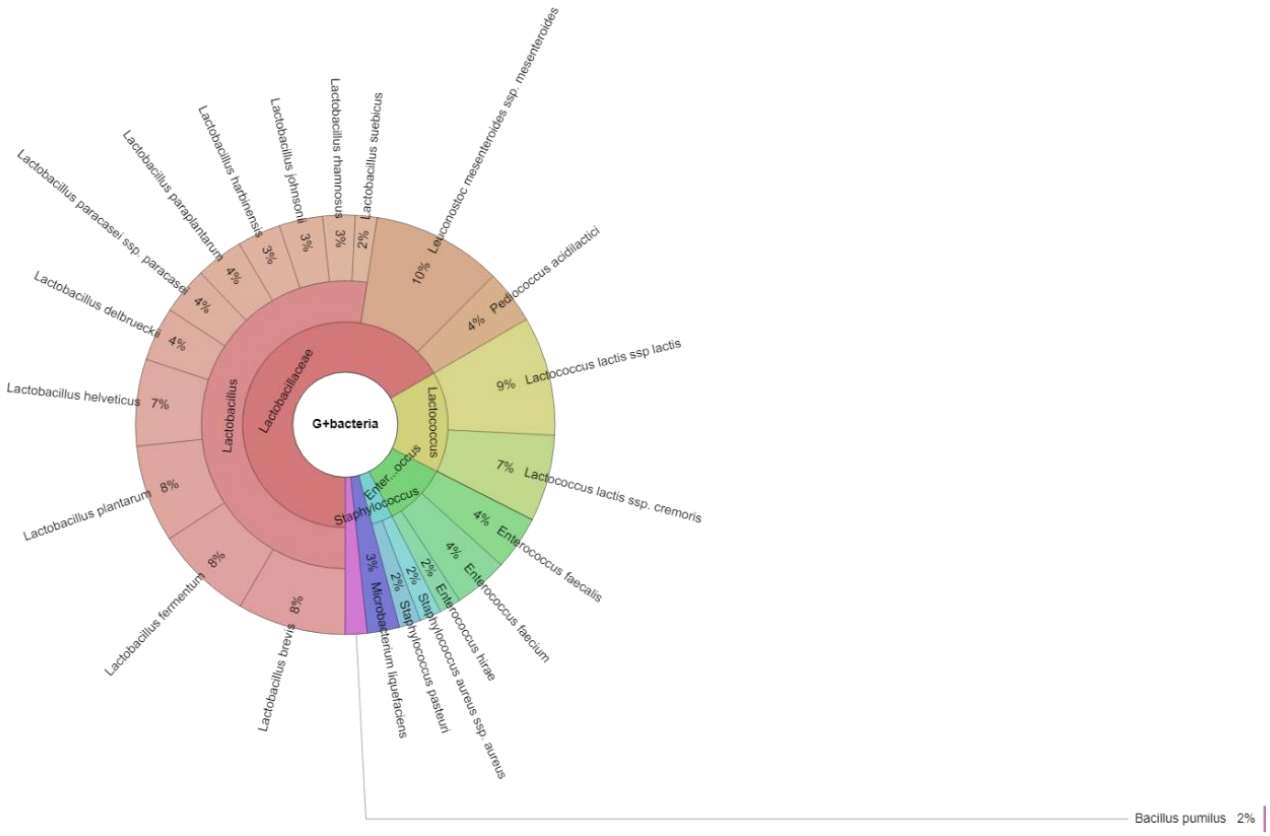

Figure 2 Gram positive bacteria isolated from ewe's cheese bryndza.

Figure 3 Microscopic filamentous fungi isolated from ewe's cheese bryndza.

The distinctive flavour of bryndza cheese produced in month May is apparently composed from compounds contained in ewes' milk and from the products of fermentation of the substrate by microflora. Principal volatile aroma-active compounds of May bryndza cheese have been characterized by **Sádecká et al. (2014)**.

Due to composition and activity of microflora is estimated to have a great impact on the flavour of bryndza cheese, several culture-based as well as culture-independent microbiological studies were carried out in this regard. Data from older culture based studies, which identified *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Kluyveromyces marxianus* and *Galactomyces geotrichum* as main components of the microflora of bryndza cheese **(Palo and Kalab, 1984; Görner and Valík, 2004; Görner, 1980)** were updated by a study of **Berta et al. (2009)**, in which a range of *Lactobacillus* spp. isolates were identified by 16S rDNA sequencing. Enterococci **(Jurkovič et al., 2006)**, staphylococci **(Mikulášová et al., 2014)** and fungal species **(Laurenčík et al., 2008)** were cultured and identified in bryndza cheese. Culture-independent studies **(Chebeňová-Turcovská et al., 2011; Pangallo et al., 2014)** provided information on the diversity of bacteria and fungi and its dynamics during the production of bryndza cheese. In the production of bryndza cheese, also interactions between lactic acid bacteria and *Galactomyces*/*Geotrichum* group **(Hudecová et al., 2011)** and competition between lactic acid bacteria and coagulase-positive staphylococci **(Medveďová and Valík, 2012)** were studied.

Although basic information on May bryndza cheese is available regarding microbiological composition as well as aroma-active compounds, most of the previous experiments were done on a limited geographical basis, sometimes with products of just one factory. In order to obtain a more reliable and representative view, this study aimed to gain data for the products from the entire territory of Slovakia that is relevant to bryndza production, i.e. specified mountainous regions of Slovakia **(Commission Regulation (EC) No. 676/2008)**. In Slovakia, the presence of Carpathian Mountains creates different climatic conditions that can have influence various characteristics of the produced bryndza cheese. These can relate, in particular, to the ewe's diet in terms of different plant species composition in the pasture and, therefore, to the quality of milk used for the production of bryndza **(Ostrovský et al., 2009)** and to different temperatures at which the lump cheese is produced, which can affect the microbial consortia in the beginning of the ripening process **(Görner and Valík, 2004)**.

CONCLUSION

The aim of our study was to evaluate the microbiological quality of Slovak ewe's cheese bryndza from producers of east, middle and west Slovakia. The number of isolated group of microorganisms was accurate for the traditional cheese produced in Slovakia. Totally 1175 isolates of bacteria with score more than 2 were identified with MALDI TOF MS Biotyper.

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INFLUENCE OF REDUCTION ON ADHESIVE PROPERTIES

Igor Stadnyk, Volodymyr Piddubnyi, Svitlana Krsnozhon, Nataliia Antoshkova

ABSTRACT
An analytical analysis of the action of rolls on the medium and its behaviour at adhesion bonds with its surface are carried out. The methods and means of carrying out researches on determination of surface roughness are offered, and the experimental setting for determining the adhesion strength is developed. To reveal the essence and understanding of the general research execution, a number of hypotheses for the determination of adhesion are given and a generalized approach to the definition of adhesion is given. The physical nature of the influence of the roughness of the roller surface on the injection of the dies depends on the shape and angle of roughness, the application of mechanical forces, the degree of its previous dispersion (recipe) and its physical and mechanical properties. The nature of the contact interaction of the dough with the rough surface of the roller working organ in the injection nozzle of the fumigation machine is established. Violation of these mutual relations leads to the production of poor-quality products and a reduction in the efficiency of the machine. The contact area of the adhesive and the component forming work for overcoming the adhesion and deformation of the environment in determining the criteria influencing the process according to each particular period of the deformation stage are substantiated. The obtained data give an answer to a number of questions about the possibility of interaction of the surface of the working bodies from the environment. On the basis of their data, the actual change in the contact adhesion in the roller unit of the molding machine with a comparative analysis of existing ones with the newly designed design is considered. It is established that in order to provide a constant area of actual contact, which contributes to better adhesion, and, accordingly, the passage of a qualitative process of tightening, compression and pouring, the necessary condition is the consistency of the specified criteria. This means that the actual contact area $S_{f.к}$, varies from $S_{N.к}$ to S_c depending on the ratio of parameters.

Keywords: dough; adhesion; adhesive; substrate; forming channel; deformation; stress

INTRODUCTION
In food technologies, in the preparation of raw materials, the receipt of semi-finished products, finished products, their storage is important interaction product with various moving and stationary surfaces. Such interaction, as a rule, leads to the adhesion of the product to the surface of the working bodies, working chambers of technological equipment, as well as structural and technological materials etc. In technology, the phenomenon of sticking is called adhesion **(Zimon and Yevtushenko, 1985)**.Adhesion of the food masses is often undesirable. Oftentimes one has to face the phenomenon when the dough is sticking to the surface, and when it is removed, the part remains on the surface of the latter. This results in loss and deterioration of the semi-finished product and its appearance. Consequently, it negatively affects the efficiency of the use of equipment. The quality of the product, leads to increased costs of raw materials and energy resources, complicates the sanitary conditions of enterprises. In the previous part of our research, an example of the effect of adhesion is given. Effect of adhesion in the formation of bagels, drying on a molding machine, where the surface of roller working bodies is rough, has grooves, grooves. This is evidenced by the sticking of the dough to the surface of the roller working bodies of the molding device **(Stadnyk et al., 2018)**.With the wide introduction into food production of modern automated, integrated mechanized lines, when the processing speed of the non-Newtonian food masses has significantly increased and new building materials are widely introduced, there is always a need to study the strength of adhesion, modelling processes. Modelling of technological processes is directed to the separation zone, considering both the type and condition of the surface, and the structural and mechanical properties of the non-Newtonian food masses. Processes associated with the adhesion of structured food masses, such as dough, baking and flour confectionery products, are still poorly studied and especially difficult to model. This phenomenon was investigated by many inventors who revealed the essence and possible ways of decreasing adhesion, its determination with the help of theoretical and experimental studies and was substantiated by mathematical modelling. Therefore, in our opinion, to

reduce the negative impact of adhesion on technological processes can be by means of a comprehensive study of this phenomenon, i.e., based on the application of modern methods of simulation, analysis of processes in the contact area, the product -product.

ANALYSIS OF LATEST RESEARCHES

The adhesion of the elastic-plastic food masses is realized at the boundary between the two solids. Elastic-plastic bodies have abnormal viscosity, which varies depending on the shear stress, mass properties and other factors. The reason for the variability of viscosity is the peculiarities of the structure of elastic-plastic bodies. Adhesion as a superficial phenomenon arises at the boundary of the distribution of two phases of heterogeneous condensed bodies: the food masses – one phase, the contact surface - the second phase **(Moriarty et al., 2011)**. Superficial properties of the food masses, in particular, adhesion, depend on the bulk properties of the masses themselves. The latter determine the contact area of two bodies, which affects the amount of adhesion and its consequence, which characterizes the condition of the surface after removing the adherent mass. The separation of material from a solid contact surface may have adhesion (the boundary separates through the surface of the contact surface), cohesive (the fringe is contained in the product layer) and mixed **(Hoevar et al., 2014)**. Adhesion is due to various forces and connections by nature, they can be divided into two groups. The first group of forces is manifested in the convergence of two bodies and in the absence of contact between them, when there is a gap of a certain magnitude. The same forces act after the violation of the contact of dissimilar bodies and cannot exist in the absence of contact.As a result of the works under consideration **(Stadnyk et al., 2019)** it was established that the structural parameters of the rolls are directed to ensure the flow of the dough during its alignment and redistribution in the volume of its mass due to the smooth drag, transport and injection in the gap between them. Although the process of injection of medium (dough) with rollers at first glance seems simple, but the construction of its mathematical model and the search for the basic calculations of dependencies is quite complicated. Now there are a number of solutions for this problem, which are based on simplification of the actual process and do not consider the influence of the elastic-viscous and plastic structure of the dough and the action on it of the pressure fluctuations. Due to the fact that the pressure of the dough on the roller working bodies is transmitted on a normal basis, on the basis of the applied working pressure and the diagrams of its change over the length of the working chamber, one can determine the forces acting on them in the zones of power and pumping. **(Stadnyk et al., 2018)**, grain storage.Surface adhesion is considered to be a complex physical and chemical process that depends on surface properties such as topography, roughness, hydrophobicity, chemical composition and surface energy; the initial amount of the medium, its size, temperature and pH of the environment, etc. **(Hoevar et al., 2014; Whitehead and Verran, 2007; Merritt and Yuehuei, 2000)**. However, among many of the factors that influence the process of adhesion, researchers **(Whitehead and Verran, 2007; Merritt and Yuehuei, 2000)** believe that surface properties play a major role. As a result, three theories of microbial adhesion to the surface have been proposed: thermodynamic, DLVO (Deryaguin-Landau-Verwey-Overbeek) theory and the theory of extended XDLVO **(Hoevar et al., 2014; Whitehead and Verran, 2007)**.The thermodynamic theory is based on the fact that when the particles are attached to the surface there is a change in the total free energy of Gibbs (the energy, which is determined in the closed system). This energy is calculated by the equation of Lifshitz-van der Waals and the acidic interactions of Lewis **(Hoevar et al., 2014)**.

$$G_{ADN} = \Delta G_{LW} + \Delta G_{AB}$$

Where:

ΔG_{ADH} – change in the total free energy of Gibbs involved in adhesion; ΔG_{LW} – the change in the total free energy of Gibbs and the forces of Lifshitz-van der Waals; ΔG_{AB} – change of free energy of acidic main forces of Lewis.

The thermodynamic theory assumes that adhesion is always a reverse and distance-independent process. This theory does not determine the influence of surface charge and the concentration of electrolytes in the environment. It is believed that this theory is most accurate when working with uncharged surfaces or in the presence of a large number of electrolytes in the medium **(Hoevar et al., 2014)**. The theory of DLVO is based on the thermodynamic theory, and also suggests that adhesion is the sum of interphase energies. This theory believes that colloidal particles of a disperse system can easily unite with each other until contact of their liquid diffuse shells occurs.

$$U^{DLVO} = U^{LW} + U^{EL}$$

Where:

U^{DLVO} – full energy interactions; U^{LW} – the energy of the forces of Lifshitz-van der Waals; U^{EL} – electrostatic energy interactions.

The theory assumes that adhesion can be reciprocal and depends on distance. It is most accurate when electrostatic forces prevail, but it is limited in the case of ignoring the effect of polar interactions **(Hoevar et al., 2014)**. In order to more accurately model microbial adhesion, the theory of XDLVO, based on the thermodynamic and DLVO theory, was proposed. According to this model, it is assumed that the adhesion is the sum of the forces of Lifshitz-van der Waals, the electrostatic and free energy of the acid-base forces of Lewis.

$$G_{ADN} = \Delta G_{LW} + \Delta G_{AB}$$

Where:

U^{DLVO} – full energy interactions; U^{LW} – the energy of the forces of Lifshitz-van der Waals;U^{EL} – electrostatic energy interactions; U^{AB} – the energy of the acidic main forces of Lewis.

As in the case with the DLVO theory, the XDLVO model believes that adhesion can be reciprocal and depends on distance.However, researchers **(Hoevar et al., 2014; Whitehead and Verran, 2007)** believe that all three theoretical models, which aim to reveal the essence of adhesion to the surface, are designed for an ideal colloidal system.

Scientific hypothesis

In production conditions, adhesion is a much more complicated process and its attachment to the surface may occur in different ways. Therefore, in our view, the process of adhesion to the surface in practice often differs from the above described theories. This is due to the fact that the surfaces of solid materials are exposed to various contacting media, adsorb organic and inorganic substances, thus forming a conditioning layer, to which the attachment of the contact medium comes. In the future, under the action of the driving forces, the formed air conditioning layer changes the physical and chemical properties of the surface, and this affects the process of adhesion.

On the basis of the above, we consider that the adhesion of the medium to solid surfaces is a two-phase process, which consists of the initial inverse (physical) and the next irreversible (molecular or cellular) phase. Adhesion to the solid surface can also be passive or active, which depends on the driving forces and transport of cell media on the basis of gravity, diffusion, or hydrodynamic forces. In addition, the process of adhesion is influenced by the physical and chemical properties of the medium, phase composition and surface roughness.

Therefore, when studying adhesion, we pay attention to the surface roughness and the parameters of the topography. Thus, proceeding from this, the process of adhesion is closely related to the amplitude surface parameter (roughness) and its spatial changes, which are characterized by morphological features of the surface (topography). Therefore, the theory of attachment of the medium to the surface should consider mainly the physical and chemical aspects of the surface of materials, and to a lesser extent, pay attention to the morphological and physiological features of the medium.

MATERIAL AND METHODOLOGY

A dough with a moisture content of 33%, for high quality wheat flakes on pressed yeast, was prepared in an opaque manner with a fermentation time of 60 minutes at a temperature of 32 – 33 °C. The quality of the pressed yeast corresponds to the DSTU. Characteristics of wheat flour:

- mass fraction of moisture,% – 14.5;
- the content of raw gluten,% – 28;
- resistance gluten compression on the device IDK-1, per.pril. – 54;
- gluten stretch, cm – 14.

The study of the dough injection process was carried out on the molding machine B-54 of the confectionery factory (Ternopil). It is known from work **(Zimon and Yevtushenko**, **1985)** that the adhesion is determined by separating the medium from the surface, measuring the separation effort. In this effort, the adhesion resistance of the medium is calculated. Therefore, the adhesiveness of the Fvd (equal to the ratio of the fracture effort of the Fvd model to the area of the nominal contact Shk) depends on the size of the surface and adhesion, the conditions of contact and the separation of the dough. Adhesion was seen as a process that occurs in time when the surfaces of two heterogeneous bodies come into contact or are violated. For the quantity that quantitatively evaluates the adhesion, the work of the separation (strength) and the unstable process of filling the medium (dough) of the groove surface of the roll at the pressure of the previous loading Pk were used.

The problem that complicates the determination of adhesion strength is the establishment of the actual contact area. After all, the size of the area of actual contact is influenced by many factors: normal pressure, the nature of the contacting bodies, as well as external factors - temperature, tenseness, duration of the previous load, speed of growth separation effort. These factors have a different effect on the change in the actual contact area.

An analysis of existing methods of studying the adhesion properties of food products showed that all the methods considered have their advantages and disadvantages. In this regard, an experimental device was developed for the study of adhesion properties of the dough consisting of vessel 1 (Figure 1), plate 2, rod 3, cargoes 4, measuring vessel 5, capacity 6, flexible hose 7, valve 8, a cable 9, a pulley 10, a tripod 11, a guide 12, and a valve 13.

This installation worked as follows. A bowl of uniform dough was placed in the vessel 1, on top of which a wide plate 2 with a rigidly fixed rod 3 was installed. The previous loading was carried out by measuring loads 4 which were mounted on the plate 2. In a measuring vessel 5, a liquid from the container 6 was fed by means of a flexible hose 7 and thus created the separation effort that was transmitted to the plate 2 by means of a rope 9, which is one end attached to the rod 3, and the other to a measuring vessel 5. In the vessel 6, a constant level of liquid was maintained. The valve 8 made it possible to adjust the flow rate of the liquid to the measuring vessel 5 and thus adjust the rate of application of the force to the plate 2.

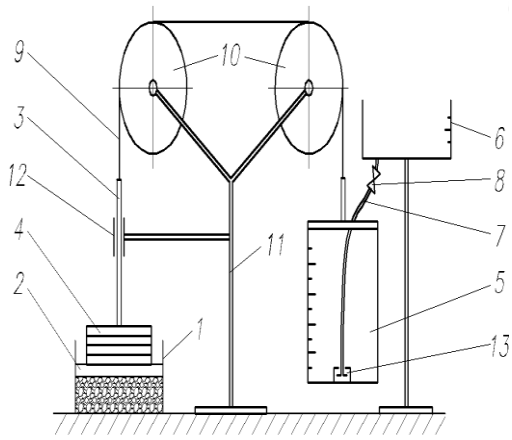


Figure 1 Scheme of the installation for the study of adhesion propertiesprotein dispersed phase:1 – a vessel; 2 – plate; 3 – stock; 4 – cargoes; 5 – measuring vessel; 6 – capacity; 7 – flexible hose; 8 – valve: 9 – cable: 10 – pulleys;11 – tripod; 12 – guiding; 13 – valve.

The change in the direction of the force by 180 ° was carried out with the help of pulleys 10 which are attached with the possibility of rotation around their axis on the tripod 11. As a previous load so and the separation effort acted at right angles to the plate 2.

This was provided by means of a guide 12 rigidly attached to the tripod 11. After the complete separation of the plate, the 2-meter vessel 5 moved down and the valve 13 blocked the feeds in the liquid from the vessel 6. The separation effort was determined through the volume of the liquid that was in the measuring vessel 5 after the plate was detached.

The developed experimental setup allowed to regulate parameters that influence the adhesion interactions: the pressure of the previous loading (0 – 10 kPa), the separation effort (0 – 100 N), the rate of growth of the separation effort (0.2 – 2 $N.s^{-1}$) and contact area

The quarrying of the experimental set-up was to determine the force to be applied to rod 3 to ensure that the plate 2 is detached from the bottom of the vessel 1 provided there is no layer of the protein disperse phase in vessel 1.

The dough was applied uniformly to the bottom of a rigid vessel with cylindrical walls in such a way that the height of the layer was 1.2 x 10^{-2} m. The area of the bottom of the vessel corresponded to the area of the plate and amounted to 7.8 x 10^{-3} m^2.

Adhesion strength was determined by substituting data obtained during the experiment into the following formula:

$$P_a = \frac{F - F_0}{S_k}$$

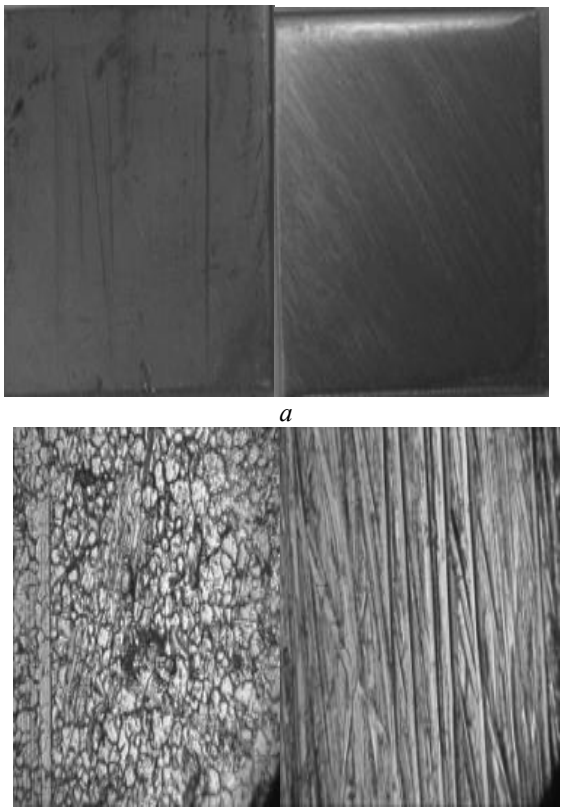
Where:

F_0 – separation effort in the absence of the test material in the vessel, H.

Methods of study of roughness of a surface

Experimental researches were carried out using modern technical and standard methods (determination of surface roughness of steel), microscopic (light and electron microscopy of the process of formation and degradation of dough residues), spectrophotometric (optical density (density), mathematical and theoretical modelling, statistical). Used plates of stainless corrosion-resistant nickel-chrome austenitic steel in the size of 30 × 30 mm and 5 mm thick, with roughness of the surface R_a = 2.687 ±0.014 microns, R_a = 0.95 ±0.092 microns, shown in Figure. 2. Their roughness corresponded to the surface of the roll.

The roughness of the surfaces of the stainless-steel plates was determined using a profiler of the mark 296. The profile of the profile (Figure 3) includes: a rack 3 for installing parts with a diamond measuring needle, a drive 1 for moving the sensor along the measured surface and an electronic unit 2 for control and calculation roughness of the surfaces. The measured part is mounted on the plate of the rack 3. If the part has a cylindrical shape, the prism 5 is installed on the stove plate and the measured part is mounted on the prism 5. The part is set so that the measured surface is perpendicular to the measuring plane. The nut 6 is designed to move the sensor 4 with actuator 1 along the guide rack 3 and install the diamond needle of the sensor 4 on the measured surface of the part.



a

b

R_a = (2.68±0.014) microns, R_a = (0.95±0.092) microns, R_a= (0.63±0.087) microns

Figure 2 The appearance of the stainless plates with different roughness: a- native appearance of the plates; B-appearance of plates using microinterferometer MII-4U4.2 (increase 1500 times).

Figure 3 Ploofilometer mark 296.

Electronic block 2 is executed in a desktop version. On the block front panel there are: a digital scoreboard for the measured Ra value; indicator of the working area; power button.

Statistical analysis

Considering the chaotic interaction of the dough during its displacement, where the change of interaction occurs between the broad-walled surface in the surface of the roll, the task planning experiment with the use of a full factor experiment of the second order is compiled. With two factors, the model of the experiment's function has the form:

$$y = f(X_1 X_2)$$

According to the results of the experiment, we obtain a regression equation of the second order.

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + b_{12} x_1 x_2$$

For experiments a plan with corresponding matrices of experiment planning with the number of experiments and boundaries of factor changes has been drawn up. The matrix is a list of options taken in this series of experiments. Independent variables were selected from the analysis of the nature of the effect on the change in the contact angle of the dough with the roll (forums number 5). As a parameter of optimization, the side a and the contact area of the broadened surface S are used respectively.

X_1 – side length **a**, mm;

X_2 – the contact area of the broad surface **S**, mm^2. Experiments were carried out on the basis of mathematical planning. Determining which factors influence the change in the angle of contact, we determine their level variations and the step of variation. The main factors and their variation equation are given in Table 1.

Output parameters were:

$У_1$ – change in the angle of interaction of the dough in height of its movement in the gap between the burrs. The displacement height of the test mass was recorded by the visual control method on a scale applied to the working roller;

$У_2$ – change the angle of interaction of the dough on its contact area on the roll surface. The weight of the test was fixed by the method of visual inspection according to the photographs.

Using the obtained data of the regression coefficients, we make a regression equation for $У_1$ and $У_2$

$$y_1 = -54.39 - 3a + 12.07S + +0.59a^2 + 0.21aS - 0.33S^2$$

$$y_2 = -17.43 + 0.8a + 3.13S + 0.11a^2 - 0.09aS - 9.9S^2$$

The analysis of the impact of the surface roughness of the roll confirms our opinion about the activity of adhesion at the injection stage and its dependence on the driving forces. From the graphical dependencies of Figure 5 – 8 it is quite clear about the influence of the parameters a and S on the process flow. Therefore, the change in the angle of interaction of the dough in its height of movement (Figure 5) depends significantly on X_2 – contact area and quite significantly depending on X_1 – the length of side a (the basis of latitude).

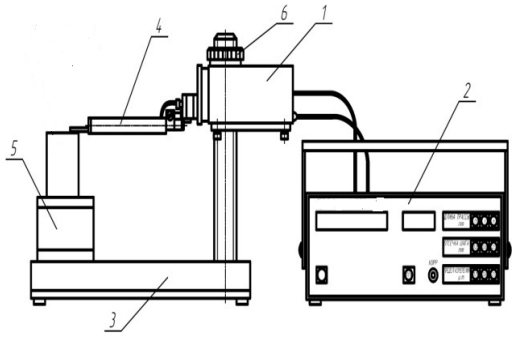


Figure 4 General view of the profile mod. 296: drive – 1; block electronic – 2; rack – 3; sensor – 4; prism – 5; nut – 6.

Table 1 The main factors and their variation equation.

Characteristics of the plan	Variable factors	Variable factors
	side length **a** X_1, mm	the area of contact of the broader surface **S** X_2,mm^2
Basic level, $X_1^{(0)}$	4.5	12
The step of variation	1.5	3
Lower level $X_1^{(-)}$(-1)	3	9
Upper level, $X_i^{(+)}$(+1)	6	16

Table 2 The experiment plan and its results.

X_1 (a, mm)	X_2 (S, mm^2)	$У_1$	$У_2$
3	9	30	5.6
4.5	9	35	7
6	9	40	9
3	12	45	9.8
4.5	12	50	10
6	12	64	10.8
3	16	60	11
4.5	16	68	11.6
6	16	75	12.4

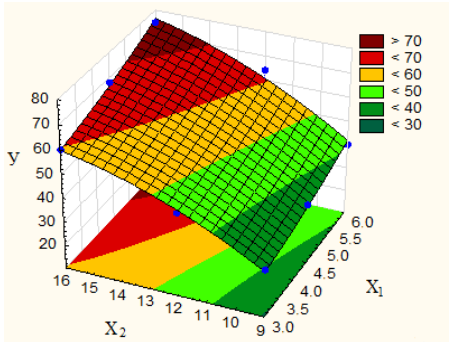


Figure5 Two-dimensional section of the surface of the response as a function.

The optimum values are within the range of 50 – 600 at the values a = 4.5 – 5.5 mm, S = 11 – 13.5 mm^2. To change the angle of interaction of the dough along its contact plane $У_2$ – the optimal values are at the corner 6 – 9^0 at values a = 5 – 6 mm, S = 10.5 – 12.5 mm^2.

Thus, the surface of the roll really creates conditions for the movement of the mass of the dough both on its surface and in the layers placed in the working chamber.

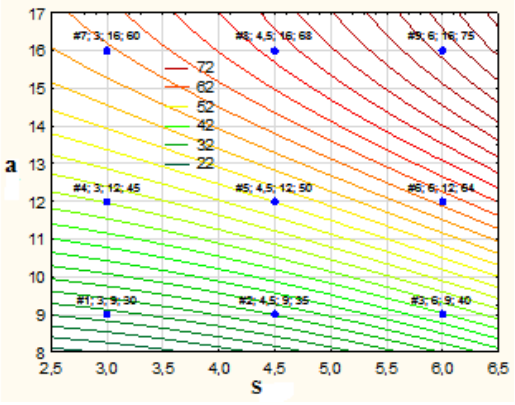


Figure6 Surface response $У_1$ = f (a, S).

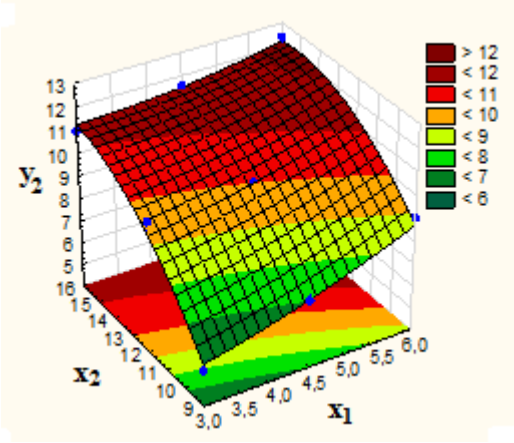


Figure 7 Two-dimensional section of the surface of the response as a function $У_2$ = f (a, S).

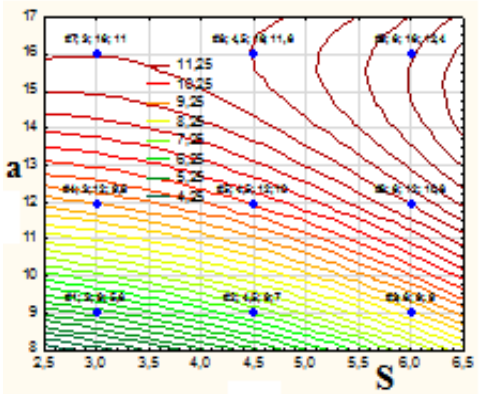


Figure 8 Surface response $У_2$ = f (a, S).

RESULTS AND DISCUSSION

The formed adhesion on the surface of the roll consists of an inhomogeneous structure, resulting in a gas concentration gradient, in particular, reducing the amount of oxygen from the periphery to the depth of the environment, pH gradients and temperature. Such gradients of functioning provide contact between the two bodies, resulting in phenotypic resistance to abrupt change in environmental factors. Data from the scientific literature **(Hoevar et al., 2014 Stadnyk et al., 2016; Gorbatov, 1979)** indicate that when studying adhesion, it is necessary to approach complexly and consider, along with roughness, the topography of the surface, as these quantities are interdependent. Consequently, the adhesion process is closely related to the amplitude parameters of the surface (roughness) and its spatial changes, which are characterized by morphological features of the surface (topography).

Studies on the effect of terrain and surface roughness on adhesion are not straightforward. Thus, according to **(Stadnyk et al., 2018; Nikolaev, 1976; Jullien et al., 2003)**, there is a correlation between surface roughness and adhesion, with the attachment of the medium to the surface increasing with increasing roughness. However, other studies indicate **(Kolari, 2003; Kukhtyn et al., 2017;Langsrud et al., 2016)** that there is practically no correlation bond between the roughness of the surface of the stainless steel and the roughness range of 0.01 to 3.3 μm. It has also been found that adhesion on surfaces with high roughness reduces the efficiency of heat transfer in heat exchangers by about 15% **(Kukhtyn et al., 2017)**.

Therefore, the conflicting data obtained by scientists regarding the effect of the surface roughness of stainless steel on the adhesion process are obviously related to the experiments under different conditions using different media, materials and methods of study. However, scientists have concluded that such elements of the surface topography as scratches, cracks, holes, protrusions, cracks play an important role in the adhesion process **(Kolari, 2003**; **Monds and O'Toole, 2009; Moons and Michiels, 2009; Zogaj et al., 2003)**.

The results of the analytical review and the conducted research and statistical modelling made it possible to approach the development of methods for determining the surface and adhesion strength by mathematical modelling. Based on the above and the data processing, two mathematical modelling approaches are proposed that can be used to determine the adhesion strength and strength.

Method of determining the wide surface of the roll

The results of the computational experiments allowed to investigate the effect of changing the angle of roughness of the roll surface on the interaction with the dough. From the studies it is clearly seen that the angle of roughness of the surface when interacting with the dough affects the adhesion properties. This approach allows us to determine rational design parameters that will contribute to the intensification of the dough injection process. Based on previous studies **(Stadnyk et al., 2019)**, when studying adhesion, it is necessary to approach the complex and consider, along with the roughness, the topography of the surface, since these values are interdependent. The surface

roughness refers to the two-dimensional surface of the material and is usually described as arithmetic mean roughness (Ra) and average square roughness (Rq). At the same time, the topography has three dimensional parameters and describes the elements of the shape of the surface. Under the strength of adhesion, adhesion must be understood as a function of factors such as separation speed, duration and pressure of the previous contact, flat contact, etc. It is necessary to distinguish between the strength of adhesion, which is measured in H (Newton), and the strength of adhesion – $N.m^{-1}$.

Interaction on the boundary of two phases, that is, the dough and the surface of the rolls, occurs from the first seconds of the molding machine. Therefore, the phenomenon of wetting the roller surface is related to the ratio of surface tensions (σ) of the adhesive and substrate. To achieve wetting on the surface of a well-adhesive roller, it must be ensured that the surface tension of the substrate is greater than the surface tension of the adhesive. This will accelerate the process as a whole with a corresponding reduction in energy costs.

The studies carried out by the authors found that the strength of the adhesion of the dough at speeds of its separation from the roller working body of the molding machine was not determined more than 1 $m.s^{-1}$. This is due, first of all, to the lack of simulation of the existing processes of formation, transport of the dough, which is mainly due to relatively small speeds of movement of the working bodies of the molding machine.

Power interaction of the medium with a roll occurs on its surfaces after the discrete injection of the mass of the test. Since the method of formation and the shape of the interaction profiles of the dough with the surface of the roll has a significant impact on the quality characteristics and the injection process, a number of comparative studies have been carried out to determine the rational roughness section of the roll.

To increase the force of adhesion between the viscous medium and the leading roller working body is possible by increasing the angle of coverage in accordance with the well-known law of Euler:

$$S_{nab} = S_{nag} e^{\alpha f}$$

Where:

S_{nab} and s_{nag} – respectively, tightening at the points of the runoff of the medium and its coincidence with a rough surface; α – the angle of coverage of the medium; f – coefficient of friction in a pair of materials.

The change in the angle of coverage of the medium is achieved due to the geometric orientation of the tightening and its injection. The problem of geometric synthesis of the system with an increase in the angle of coverage of the medium is solved on the basis of geometric bonds. Consider cases of power interaction, in which the line of vertices of roughness of a roll:- parallel to the vector of injection velocity $\upsilon_p$and the circular power R_{KB} (angle α = 0 °);

- not parallel to the velocity vector injection υr and circular power R_{KB} (angle α >0°).

The angle α depends on the lifting of roughness (Figure 6). Roll movement in the middle during its injection can be divided into 3 components:

- rectilinear horizontal in the direction from the axis of the roll to the inner surface of its body, due to centrifugal force Pvb;

- rotational horizontal in the direction of rotation of the roll due to the force of the knee P_{KB};

- straight vertical from the force of gravity on the dough layer of the dough that is above it $P_{TG.}$

Due to these movements, the friction forces (adhesion) appear on the upper and lower front roughness of the roller surface. The three components of the dice movement described above correspond to the coordinate axes of the XYZ Cartesian coordinate system (Figure 9). That is, the direction of the axis OX (vertical axis) coincides with the direction of gravity, $P_{TG.}$, OY (horizontal axis) with the direction of action of the centrifugal force P_{VB}, OZ (orthogonal) with directional velocity υ_{KB}, circular force P_{KB} and the forces of compression of the dough P_{CT}. With a steady state of work, we assume that the velocity of the circle υ_{KB} will be equal to the pumping speed υp ($\upsilon_{KB} = \upsilon_{н}$).

In the case of α = 0 °, the vertical coordinate plane XOY will be perpendicular to a plane passing through lines of roughness to its front surface. If α >0 °, then the angle between the above-mentioned planes will be 90 °±α.

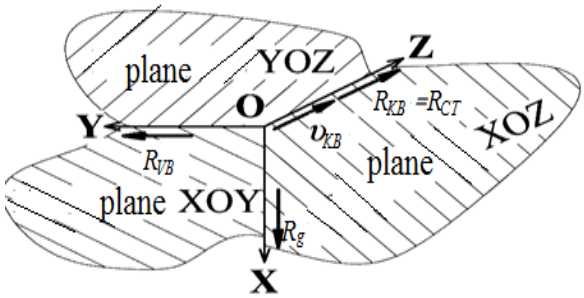


Figure 9 Coordinate system, which considered power interaction: R_{VB}– centrifugal force; Rg– gravity force; R_{KB} – the force acting on the dough; P_{CT} is a compressive force; υ_{KB}– circular speed.

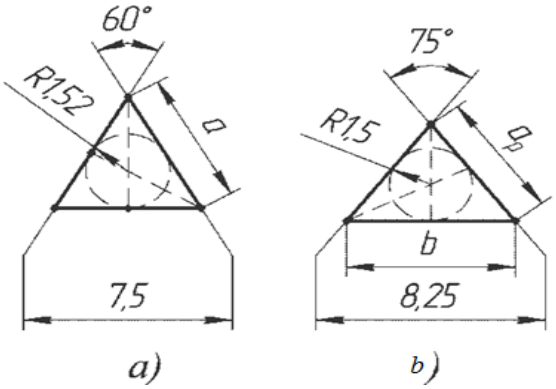


Figure 10 Cross-sections of roughness of different profiles with an area of 1.2 mm^2 each: a) triangular, equilateral; b) triangular, equidistant.

For comparison, two triangles were chosen (an equilateral triangle, an equilateral triangle with an angle at

the vertex of 75, cross-sections of roughness with an area of 1.2 mm^2 each (Figure 10).

For an equilateral triangle S, the length of a side is determined by the formula:

$$a = \sqrt{S\frac{\sqrt{3}}{4}}$$

For an isosceles triangle, the area (S) will be determined by the formula:

$$S = \frac{1}{2}a_p b sin\alpha \quad (4)$$

Where:
a – side of the triangle; b – the basis of the triangle; S – is the angle between the side and the base (for an isosceles triangle with an angle at the apex 75 ° α = 52.5 °).

We will define the projection theorem ***b***:

$$b = 2a_p cos\alpha \quad (5)$$

Substituting equation (5) into formula (4) and expressing ***a***, we obtain it:

$$a_p = \sqrt{\frac{S}{cos\alpha\ sin\alpha}}$$

Where:
S =1.2 mm^2 → *ap* = 0.498 mm, *b* = 0.606 mm.

Method of determining adhesion strength

At the moving surface of the rotary rollers, a general work is performed which consists of elastic forces and changes in the contact of the rolling dough: $A_z = A_{pr} + A_k$.

Work of elastic forces:

$$A_{pr} = S_{nk}(l_1 - l_0)$$

Specific work 1 kg of dough (in J) can be calculated by the formula:

$$A_{pr} = \frac{A_z}{m_{sr}\ 10^3}$$

Where: m_{sr} – average weight of dough between surfaces of rotating rolls, kg:

$$m_{Sr} = \frac{V}{v}$$

Where: υ – specific volume; V– camera volume.

The work that is spent on changing the contact of a moving layer of the medium with the surface of the working chamber and rollers to overcome the adhesion and deformation of the medium A_d, will be:

$$A_V = A_{ad} + A_d = F_V dx = F_{ad} dh + A_d dh$$

Where:
F_{ad}, A_d – the efforts of adhesion and deformation;
h – the thickness of the medium on the surface of the roll when the separation of its layer on the subsequent process -formation;
F_{vid} – the effort of separating a piece of medium from the surface of the roll.

Consider the components forming the work of the separation:

$$F_{ad} = \int_o^i frld = f_{ad} rl^2 \quad (6)$$

Deformation of the environment is determined:

$$F_d = \tau_0 \frac{V_v}{h} rl \quad (7)$$

Where:
V_v –the speed of separation of the medium from the surface of the roll under the action of external forces; τ_0 – tangential stresses; η – plastic viscosity of the medium; r – dough layer on roller; l – the length of contact of a part of the working body.

Given the length of the contact area, we obtain the expression:

$$F_d = \tau_0 \frac{V_v}{h}\ rl$$

Work determined by the separation effort is spent on overcoming adhesion F_{ad} and deformation of the medium, when exiting through a rectangular molding surface between rotating rolls F_{def}

$$F_V = F_{ad} + F_d \quad (8)$$

When studying the process of dough injection, one of its conditions was to change the gap between the rolls and the angular velocity of their rotation. Knowing the mass of the dough, the area of its contact with the surface of the mixing drum, with the help of the proposed method and computer symbolic mathematics, the strength of adhesion is determined.

Initial speeds are selected at three values of the crack (δ = 20 mm; δ = 25 mm; δ = 30 mm). Trajectory of the mass of the test for three cases in the car chamber: $v_0 = 0.18$ m.s^{-1}; $v_0 = 0.32$ m.s^{-1}; $v_0 = 0.4$ m.s^{-1}. On the dash movement there is an impedance which is expressed by the coefficient of resistance K. For the three clearances we make the equation of spline approximation:

$$v_0 = 0.18 ms^{-1}$$

$$K = 4.58x^2 - 3.25x + 2.7$$
$$v_0 = 0.32 ms^{-1}$$

$$K = 3.65x^2 - 2.9x + 2.69$$
$$v_0 = 0.4 ms^{-1}$$

$$K = 2.29x^2 - 2.26x + 2.73$$

Where:
v_0– speed of the dough movement at the output of the rollers, with different values of the gap;
K – factor of resistance.

The graph of the relationship between the coefficient of resistance K and the length of the dough movement at the output of the rollers (x) is shown in Figure 11.

Experimentally determining the value of length (x), substituting it into an approximation equation, one can determine the coefficient of resistance K, which is the sum:

$$K = K_{\Pi} + K_{ad}$$

Where:

K_n– component of the resistance coefficient considering the air resistance;

K_{ad}– part of adhesion.

The air resistance coefficient is 0.11.

Consequently, considering the trajectory of the dough movement at m.s^{-1} the most favourable (gap of 30 mm), we will substitute x = 0.3 into the approximation equation:

$$K = 2.29(0.3)^2 - 2.26 \cdot 0.3 + 2.73 = 2.26$$

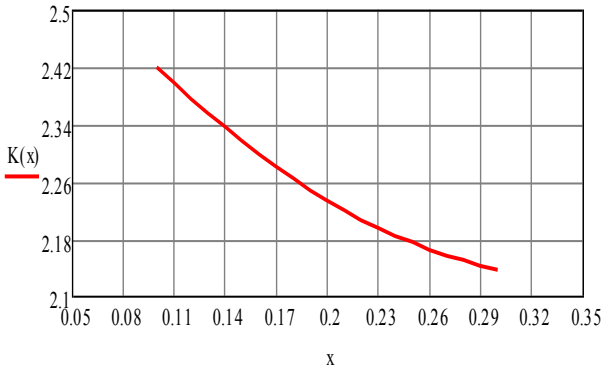


Figure11 Charts of functions K (x) for an initial speed of 0.18 m.s^{-1}.

We find the coefficient of adhesion, which in our case will be:

$$K_{ad} = K - K_n = 2.26 - 0.11 = 2.15\frac{Hc}{m}$$

In accordance with the chosen model of motion, we consider that due to adhesion, the strength of resistance appeared, directed against the movement of the mass of the test, equal to:

$$P_{on} = K_{ad}\frac{\partial x}{\partial t}$$

Since x-axis is chosen for calculations, then:

$$\frac{\partial x}{\partial t} = v_0 \cdot cos\alpha$$

Substituting this equation in (в), we obtain:

$$P_{on} = K_a v_0 cos\alpha$$

Knowing the magnitude $K_{ad}, v_{0,} cos\alpha$, we will define:

$$P_{on} = 2.15 \cdot 0.4 \cdot cos15^0 = 0.831H$$

Where:

$cos15$ – the angle between the surface of the roll and the detached moving dough during the injection process.

Area of the nominal contact

$$S = 2\pi R\frac{1}{8}(2b + \frac{2}{5}\text{R}) = 2\pi 0.11\frac{1}{8}\left(2.02 + \frac{2}{5}0.11\right) = 9.0343m^2 \quad (8)$$

Where:

R – roll radius;

b – roller width (Figure 12).

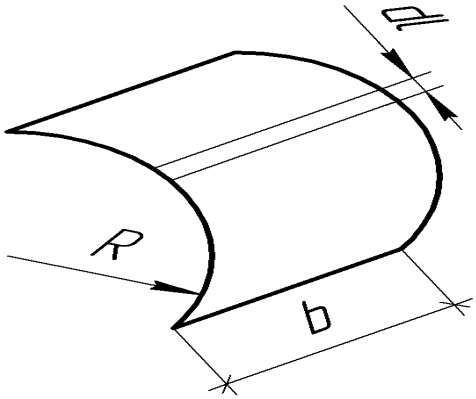


Figure 12 Scheme of Roll.

$$F_{ad} = \frac{P_{ad}}{S} = \frac{0.831H}{0.0343m^2} = 24.2\frac{H}{m^2}$$

considering relations (6) and (7) – (8), the separation effort will be in the form:

$$f_{ad} + \tau_0\frac{V_v}{h}F_V = S_{re} \cdot f_{ad} + \tau_0\frac{V_v}{h} \quad (9)$$

With these conditions, it is possible to determine the actual adhesion by the results of an adhesion test at insignificant discontinuation rates when Vv → 0.

These conditions are fully consistent with the processes taking place at the pumping stage. In addition, we know the actual contact area of the phases. Minimum actual contact area S_{nk}, formed by a contact of a dough with a roll surface, where α = 1. The maximum contact area is equal to the surface area of the roll (substrate) Sc (Figure 13).

The nominal contact area is easily determined by the geometry of the adhesive or substrate. The area of the substrate, considering the relief surface (depends on the frequency and type of its processing, Figure 13, type A) is known to us. Therefore, in the general form, the relief of the surface with the side a can be written by a double Fourier series:

$$Z = \sum_{m,n-1}^{\infty} a_{m,n} \cdot sin\frac{\pi mx}{a} \cdot cos\frac{\pi RY}{a} \quad (10)$$

Where:

Z – the value of the height of the inequalities;

a_{rs}- Fourier coefficients;

x, y – Cartesian coordinates;

m, n – Harmonic numbers.

The forces of adhesion in these cases are very small.

Expression (10) characterizes the profile of any surface. The element is selected on the surface of the areas :

$$ds = \sqrt{1} + (\frac{dz}{dx})^2 + \sqrt{1} + (\frac{dz}{dy})^2 dx \cdot dy$$

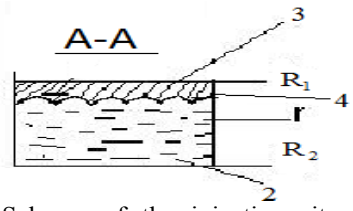


Figure13 Scheme of the injection site: 1–working chamber, 2–dough, 3–rolls; A-determination of the actual contact area of the roller working body and the dough: 2–adhesive (dough); 3 – substrate; 4 – surface of the substrate.

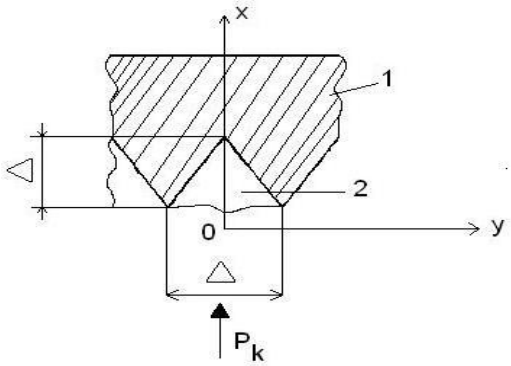


Figure14 Filling the cavity on the substrate surface 1 with adhesive 2.

Knowing the profile form, i.e. dz/dx and dz/dy, it is possible by means of integration to define Sc based on the topography of the surface. So, as the relief of the surface of the roller working body has grooves with the appropriate angle, the angle of the vertex between the groove protrusion γ = 60° at the top of the trapeze. In any arbitrarily selected slit, the relief has the form of an equilateral trapezoid (Figure 16). Then the length of the profile formed by the surface in 2 times will increase the length of the middle line of the profile. Accordingly, the surface area of the substrate will be 2 times greater than the nominal contact area.

In real conditions, the dough does not fully contact the surface of the roller working body. According to the work **(Nikolaev, 1976)**, the filling of the rough surface is proportional to the pressure and time of contact of the dough, as well as its viscosity:

$$\frac{h}{d} \sim \left(\frac{p_k t_k}{\mu}\right)^{\frac{1}{2}}$$

It can be assumed that the dough is in contact with a broad-walled surface at a contact pressure Pk. The width of the roll is characterized by the mean square value of the inequalities Rz. In this case, the cavity on the surface of the roll is filled with a dough. If the size of the macromolecules of the dough is much smaller than the slit of the surface, the law of the mechanics of continuous media propagates in the car's current and the two-dimensional flow motion passes.

In this case, the forces of inertia are small, and the forces of hydrostatic pressure, viscous friction and capillary are mutually balanced. In such conditions, to obtain the basic criteria for the similarity of the current, it is sufficient to consider the one-dimensional equation of the dough movement. In isothermal flow, when the values of the temperature of the dough and rolls are equal (this is evident from the experiments of **(Stadnyk et al., 2019)**, the equation of motion has the form:

$$Q = -\frac{\partial p}{\partial x} - \frac{1}{\rho} \cdot \frac{\partial \tau_{xy}}{\partial y} \qquad (11)$$

Where:
p – pressure;
x, y – Cartesian coordinates;
$\tau_{x,y}$– tangential tension;
ρ– density of the dough.

On the free surface of the dough, the conditions of continuity of the normal and the absence of tangential stresses are fulfilled.

We denote the radius of curvature of the adhesive on the surface of the substrate (Figure 14) Δ, then the condition of the continuity of normal stresses on the Laplace formula has the form:

$$\tau_x = \frac{\delta_{rn}}{\Delta} \qquad (12)$$

Where:
δ_{rn}– surface tension on the boundary adhesive - surface.

The rheological properties of the dough in the region of small deformation velocities, namely, when passing through the gap between rotating roller working bodies, is characterized by the Shvedov-Bingam equation **(Stadnyk et al., 2016; Believ, Egorenkov and Pleskachevsky, 1971)**,

$$\tau = \tau_0 \cdot sinj = \mu_{pl} \qquad (13)$$

Where:
τ_0– conditional yield curve;
μ_{pl} – plastic viscosity;
j – gradient of strain rate.

The tensile stress in the xx direction is determined accordingly

$$\tau_{xx} = 2B\frac{\partial v}{\partial x} \qquad (14)$$

Where:

$$B = \tau \cdot A - \mu_{pl}$$

A – the second invariant of the strain rate tensor, obtained for a one-dimensional flow from the ratio:

$$A = \frac{\partial v}{\partial x} \qquad (15)$$

Considering (13) – (15) and equation (11) and boundary conditions (12) can be written as:

$$\frac{\partial p}{\partial x} + \frac{\partial}{\partial y}\left(\tau_0 + \mu_{pl}\frac{\partial v}{\partial x}\right) = 0 \qquad (16)$$

$$2\left(\tau_0 + \mu_{PL} \cdot \frac{\partial V}{\partial x}\right) = \frac{\delta_{ab}}{\Delta} \qquad (17)$$

Replacing differentials with characteristic values

$$P \approx P_K; \text{У} - \Delta; \text{X} \approx \Delta; v \approx \frac{\Delta}{t}$$

we get dimensionless components:

$$N_1 = \frac{\delta_{AB} t_k}{\Delta \mu_{pl}}; N_2 = \frac{\tau_{0\cdot\cdot} t_k}{\mu_{pl}}; N_3 = \frac{p_k t_k}{\mu_{pl}} \qquad (18)$$

Figure 15 Photo of adhesion of the dough to the surface of the roll of a new design: 1 – roll with screw grooves; 2 – car body.

Criterion N_1, characterizes the ratio of forces of surface tension and viscous friction; N_2 analogue of Saint-Venant's criterion for conditions of non-stationary movement (flow); N_3 – takes into account the influence of contact pressure and viscous friction. Thus, completeness complements the grooves of the working body $S_{f.к}$ varies from $S_{N.к}$ to S_c depending on the ratio of the parameters of the criteria N_1, N_2, N_3. These criteria, as well as a number of others, affect the injection according to each particular period of the corresponding stage of the process. From the criterion equations, it is evident that the plastic viscosity of the dough is of great importance. At the first minutes of the process, the contacts of the medium with the surface of the roller working body pass through the basic principle of slow plastic deformation. With increased contact time with the contact pressure present, adhesion elasticity increases, mainly due to the plastic flow and is determined by the value of plastic viscosity. At the exit from the molding channel formed by the roll's criterion N_3 loses its meaning and there is a qualitative separation of the given mass of the test to the molding device. At the same time, there are two criteria N_1 and N_2 continue to affect the bulk of the dough that is on the roll, retaining its plastic properties and thereby ensuring the cleanliness of the surface (Figure 15).

CONCLUSION

To ensure a constant area of actual contact, which contributes to better adhesion and, accordingly, the passage of a qualitative process of tightening, compression and pouring, the necessary condition is the consistency of the criteria (18). This means that the actual contact area $S_{f.к}$, varies from $S_{N.к}$to S_c depending on the ratio of parameters. Therefore, to meet the requirements of the process, the gap between the rotary rolls is set at the appropriate distance to create a forming channel, which corresponds to theoretical and practical calculations. In such conditions, qualitative stages of the process in the injection site pass through large stresses of deformations. These conditions must last for a certain period of injection. Therefore, the effect of adhesion should be minimal. It is possible to change the forces of adhesive bonding only if the thickness of the dough layer is sufficiently small and in the separation from the surface of the shafts should be clean.

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COMPARATIVE ASSESSMENT OF STORAGE STABILITY OF GINGER-GARLIC AND CHEMICAL PRESERVATION ON FRUIT JUICE BLENDS

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ABSTRACT
The study aimed at reduction of wastage of fruit, encourage production, consumption and preservation of fruit juice blends using garlic ginger filtrate with health benefits as biopreservative thus providing alternatives with biological advantage over chemical preservatives (ascorbic and benzoate acids) without altering the organoleptic and physicochemical properties of fruit juice blends. The study evaluated the potential of natural preservatives (ginger, garlic and ginger-garlic filtrates) in comparison with two conventional chemical preservatives (ascorbic and benzoate acids) for fruit juice blends preservation. The juice blend used was cashew, pineapple and watermelon. In terms of flavor and mouth feel, the order of preference of the juice were the preserved with 1% garlic-ginger > 1% ginger > 1% garlic > 1% ascorbic acid > and preserved with 1% sodium benzoate at ambient temperature. Maximum decrease in pH was observed in the juice sample that had no added preservative. Generally, all the fruit blends (preserved and unpreserved), with the exception of the one preserve with 1% ginger-garlic showed growth of bacteria after one week of storage. Juice blends preserved with the 1% ginger-garlic were most acceptable compared to other preservatives. The synergistic biopreservative ability observed with the ginger-garlic may be a preferable alternative to conventional preservatives.

Keywords: juice blends; ginger; garlic; stability; preservatives

INTRODUCTION
Juices are the extractable fluid contents of cells or tissues intended for direct consumption obtained by the mechanical process from sound, ripe fruits **(Naz, 2018)**. They are non-alcoholic liquid products with diverse degree of clarity and viscosity **(Sádecká et al., 2014)**. Fruit juices are rich in lycopene, ascorbic acid and citrulline that have been reported to have protection against cancer and cardiovascular disorders. The functionality of fruit juices have been attributed to their antioxidative properties **(Okwori et al., 2017)**. Water melon is a common staple fruit in the world consumed as a dessert, fruit salad or used fo garnishing drinks **(Mohammad, 2016)**. Cashew is a hard, drought-resistant, tropical tree, widely grown primarily for its nuts. Cashew apple, the pseudo-fruit, is fibrous, juicy and weighs approximately 8 times of the nut **(Afolayan et al., 2016)**. Much (90%) of the harvest is wasted after harvesting **(Igbinadolor et al., 2017)**. Because, cashew apple has a characteristic astringent taste with biting sensation on the tongue and throat, blending it with other fruits may lessen its astringency **(Rebouças et al., 2016)**. Raw pineapple juice is an excellent source of calcium, magnesium and manganese; however, pineapple mostly consumed around the world as canned products **(Kaddumukasa et al., 2017)**. The demand for pineapple and its juice rises continually most especially due to its health benefits **(Nwachukwu and Ezejiaku, 2014).** It is usually used for blend composition to obtain new flavors in drinks. Most juice if not refrigerated has a very short shelf life **(Okwori et al., 2017)**. Ginger is a spice with characteristic flavor due combination of zingerone, shogaols and gingerols and volatile oils. Fresh ginger is composed of 80.9% moisture, 23% protein, 0.9% fat, 1.2% minerals, 2.4% fibre and 12.3% carbohydrates **(Olaniran and Abiose, 2018)**. Ginger has antibacterial effect and exhibits antifungal activity and extended the shelf life for 8 weeks in tomato paste. Ginger powder has been compared with synthetic antimicrobial agents such as potassium sorbate and citric acid in smoked fish **(Oduah et al., 2015)**. Ginger is a commonly added to beverages for flavor. Garlic (*Allium sativum*) also regarded as Russian penicillin, stinky rose, *tafanuwa* in Hausa, *ayo-ishi* in Igbo and *ayu* in Yoruba **(Neeraj et al., 2014; Olaniran et al., 2019a)**. Garlic comprises of sulphur containing compounds, the fresh bulb contains allicin, alliin and volatile oil. Garlic has exhibited antibacterial activity against Gram positive and Gram-negative bacteria **(Olaniran et al., 2015)**. Fruit juice blends produced from different fruits combines basic nutrients present in these different fruits to provide a better quality juice nutritionally and organoleptically **(Eke-Ejiofor, 2016)**. The inhibition of microbial growth and activity of

microorganisms is one of the main purposes of the use of chemical preservatives such as benzoic, sorbic, lactic and acetic acid **(Piper, 2018)**. Benzoic acid has been used in different forms as preservative in foods because of its established antimicrobial properties against yeasts and molds. They can denature protein, inhibit enzymes and alter or destroy the cell walls or cell membranes **(Reut et al., 2004)**. Current reduction in consumption of chemically preserved foods is due to consumer's awareness of the health implication of consumption of synthetic preservatives **(Pongsavee, 2015)**. Replacing chemicals with natural preservatives (bio-preservatives) which have no side effects to the consumer is of interest. To provide alternatives with biological advantage over chemical preservatives without altering the organoleptic and physicochemical properties of fruit juice blends, the need to explore natural preservatives has been highlighted recently in scientific literature. In this regard therefore, the current study aimed to apply ginger-garlic mix; exploring their effectiveness as preservatives and assessing organoleptic acceptability of the new combination in fruit juice blends using cashew, pineapple and watermelon fruit for the storage study.

Scientific hypothesis

Biopreservatives are as effective as chemical preservatives in the preservation of fruit blends. The presence of biopreservatives in fruit blends can improved their organoleptic properties, when compare to chemical preservatives.

MATERIAL AND METHODOLOGY

Preparation of preservative filtrates

Garlic, ginger and ginger-garlic filtrates were used as preservatives in this study. For preparation of the filtrates, fresh ginger rhizomes and garlic cloves obtained from a local market in Kwara State, Nigeria. Prior to use, they were washed under running water, peeled and diced into cubes separately. The respective diced cubes (100 g) were blended with 100 mL of distilled water using a grinder (Marlex Appliances PVT, Mumbai, India) for 5 min and allowed to stay for 30 min. The suspensions were then filtered and the filtrates were poured into labelled clean bottles. Garlic-ginger mix was obtained by mixing equal volume of garlic and ginger filterate and homogenized for 60 s **(Olaniran et al., 2019b)**.

The preservatives used were 1% ginger filtrate, 1% garlic filtrate, 1% ginger-garlic, 1% ascorbic acid and 1% sodium benzoate**.**

Preparation of the juice blend

For preparation of the juice blend, pineapple, watermelon and cashew fruits were used. Three kilograms of each of the fresh, ripe fruits were respectively washed under running water, drained in colanders, peeled and diced into cubes. Each juice of the edible parts of the respective fruits was extracted separately using juice extractor (Imarflex IM-3180, Quezon City, Philippines).

Following extraction, the cashew, pineapple and watermelon (CPW) juice were mixed in ratio of 10:50:40 (v/v) respectively to obtain the blends which was homogenized for 10 sec. A glass jar of CPW juice, containing no preservative was maintained as positive control. Five other separate jars were engaged with five different pretreatments.

Sensory analysis

To estimate consumers' acceptability, the following sensory attributes were investigated: aroma, color, flavor, sweetness, mouth feel and overall impression. A nine-point structured hedonic scale test (9 = "extremely like"; 5 = "neither like nor dislike"; 1 = "extremely dislike") was used for the assessment of overall acceptance of the freshly prepared juice blend, with and without the respective preservatives.

For investigation, the samples were served in a sequential manner in cups containing 25 mL of the respective juice treatments and codified with three random digits. The sensory evaluation was conducted with 45 panelists (25 females and 20 males) comprising of students and staff of a university and aged between 18 and 50 years. The inclusion criteria in being selected as a panelist on condition of regular consumption of the juice blend. Approval was granted by the University research ethics boards (LUAC-0046B).

Determination of physical characteristics

pH was determined using a pH meter (Jenway model 6505). Before use, the pH meter was calibrated using standard buffers of 7.0, 4.0 and 9.2. After calibration, pH readings were read and documented only when equilibrium pH was reached.

Titratable acidity and specific gravity were determined as described in **AOAC (2010)**. For determination of total soluble solids, a refractometer (Hanna HI 96801) was used. Values were measured in % Brix as described elsewhere **(Makebe et al., 2017)**.

Microbiological enumeration

During the storage period, microbial analysis was carried out on a weekly basis for 5 weeks, using nutrient agar (Oxoid limited, UK), De Man, Rogosa and Sharpe agar for enumeration of lactic acid bacteria incubated at 37 °C for total bacterial and lactic acid bacteria count for 24 and 72 hours respectively and potato dextrose agar incubated at 28 °C for 3 days, for estimation of fungal count, using the standard pour-plating method **(APHA, 2015)**.

Statistical analysis

All data from sensory evaluation, variation in pH, titratable acidity, specific gravity and total soluble solid of the juice blend containing different preservatives during storage were analysed using analysis of variance (ANOVA) and differences in mean values were assessed using Duncan's multiple range test. A value of $p < 0.05$ was used to indicate statistical significance. Using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) means of the replicates were all calculated and separated.

RESULTS AND DISCUSSION

All samples were acceptable in terms of color, taste, flavor and texture. In terms of flavor and mouth feel, the order of preference of the juice were the preserved with 1% garlic-ginger > preserved with 1% ginger > preserved with 1% garlic > preserved with 1% ascorbic acid > and preserved with 1% sodium benzoate at ambient temperature of blends. Addition of preservatives enhanced the colour of the juice blends with the preserved with 1% ginger-garlic having the highest mean score of 8.88. There was no significant difference (p <0.05) in the sweetness of all the juice blends and the scores were in-between 8.77 to 8.94. Combination of ginger and garlic significantly (p <0.05) improved the mouth feel of the juice blends. In terms of overall impression, the most acceptable juice blend was the one preserved with 1% ginger-garlic (Table 1). In this the study all the juice blends that was garlic-ginger preserved was most acceptable and preferred. This observation may be due tempering of the pungent taste of garlic with pleasant scent of ginger resulting from mixing of equal volume of garlic and ginger filtrates. A similar observation has been reported by **Mancini et al. (2019)**. The least preference for the garlic preserved juice blend, as observed in this study may be due to the strong pungent taste of garlic compared to that of those with ginger due to its pleasant **(Mancini et al., 2019)**. In a study on the preservation of soymilk, **Borode (2017)** however reported low preference for garlic preserved soymilk.

In all the juice samples, pH was observed to show consistent decreases with period of storage. Maximum decrease in pH was observed in the juice sample that had no added preservative. At end of the five weeks storage period, pH decreases of 31.6, 25.1, 30.8, 30.9, 20.7 and 25.1% were observed in the juice samples without preservative, preserved with 1% ginger, 1% garlic, 1% garlic-ginger, 1% ascorbic acid and 1% sodium benzoate, respectively
(Table 2).

For the titratable acidity, consistent increases were observed in the juice sample throughout the period of storage. Increases of 90.9, 133, 240, 35.7, 200 and 80% were observed in juice samples the juice samples without preservative, preserved with 1% ginger, 1% garlic, 1% garlic-ginger, 1% ascorbic acid and 1% sodium benzoate, respectively (Table 3). The consistent decrease in pH with period of storage as reported in this study is indicated to be vital to retaining the quality of tartness to the product. Decrease in pH with period of storage has been also been reported by previous investigators **(Kaddumukasa et al., 2017; Olaniran et al., 2019b)**. It is opined that that low pH could enhance the stability of bioactive compounds during storage, thus extending shelf life **(Chia et al., 2012)**.

In the case of specific gravity, increases were observed at the end of the five weeks period of storage. However, only minute increases of 8.4, 4.5 and 6.0 were observed were observed in juice samples preserved with, preserved with 1% ginger, 1 % garlic and 1 % garlic-ginger, respectively. Remarkable increase of 44.7% in specific gravity was observed in the unpreserved juice sample (Table 4). The total soluble solids in the preserved juice blends were observed to show consistent decreases with time. This observation was irrespective of the preservative used. Highest increase (12.1%) in total solids was however observed in the unpreserved juice blend at the end of the five weeks storage period. At the end of storage, increases in total soluble solids of 6.3, 3.6, 5.5, 10.5 and 9.4% were observed for juice blends that were preserved with 1% ginger, 1% garlic, 1% garlic-ginger, 1% ascorbic acid and 1% sodium benzoate, respectively (Table 5). The stability documented in the study in the values of total soluble solids and specific gravity of preserved juice blends for weeks might be due to the presence of garlic, ginger, garlic-ginger filtrates, sodium benzoate and ascorbic acid added as preservatives. The preservatives slowed down the rate of fermentation of sugars present in the juices blends to water, carbon dioxide and ethyl alcohol at room temperature during storage **(Kaddumukasa et al., 2017)**. In this study, the concentration of total soluble solid in the juice blends showed consistent increases with period of storage. Increase in soluble solids is an indicator of the rate of deterioration. The minimal increase soluble solid level observed in the preserved juice during storage could be due to decrease in the rate of conversion of organic acid to sugar thus increasing the shelf life of the juice. Similar observations have been reported by earlier researchers in related studies **(Samad et al., 2019; Rapisarda et al., 2008)**. Generally, all the fruit blends (preserved and unpreserved), with the exception of the one preserve with 1% ginger-garlic showed growth of bacteria after one week of storage. Growth was only observed in the 1% ginger-garlic preserved juice from the third week of storage. At the end of the 5-week storage period, the bacterial count in the unpreserved juice was however remarkably higher than those of the preserved samples. The growth of lactic acid bacteria was observed in all the juice samples throughout the period of storage, except the 1% garlic and 1% ginger-garlic preserved samples where growth was observed only after 2 and 3 weeks of storage, respectively. For the total fungal counts, no growth was observed for the ginger-garlic preserved juice until after 5 weeks of storage. The 1% ginger and 1% garlic preserved juice showed growth after 2 weeks and 3 weeks of storage, respectively (Table 6). Combining the garlic-ginger as preservative in the study was the most effective during storage as microbial growth was greatly inhibited. This could be as result of a synergistic effect of ginger and garlic **(Juan et al., 2017)**. It is opined that the major challenge in spoilage of fresh juice is the stability of the pH, natural microflora; chemical composition fruit juice **(Ephrema et al., 2018)**. From the results from the study, addition of 1% garlic-ginger as preservative was effective in reduction of the microbial load and other physicochemical parameters. This may be due to the presence of essential oil from garlic and ginger, which are reported to have health promoting bioactive components offering consumers health benefits **(Aneja et al., 2014; Baskaran et al., 2010)**. Thus, if 1% garlic-ginger is incorporated into industrial production of cashew apple, pineapple and water melon juice blends; it has better potential replacement for the chemical preservative during storage.

Table 1 Sensory Evaluation of freshly prepared Cashew, pineapple and watermelon blends.

Attributes	CPW	CPW-GIN	CPW-GAR	CPWGG2	CPW-ASC	CPW-SBZ
Sweetness	8.89 ±0.02^{bc}	8.94 ±0.01^{a}	8.82 ±0.04^{ab}	8.86 ±0.01^{a}	8.77 ±0.02^{c}	8.86 ±0.03^{a}
Color	8.00 ±0.01^{b}	8.64 ±0.04^{aa}	8.22 ±0.02^{b}	8.88 ±0.01^{a}	8.15±0.03^{b}	8.25 ±0.01^{b}
Flavor	7.78 ±0.03^{a}	7.75 ±0.01^{a}	7.22 ±0.03^{bc}	7.92 ±0.02^{b}	7.33 ±0.01^{a}	7.67 ±0.02^{c}
Mouth feel	7.42 ±0.01^{b}	7.44 ±0.02^{b}	6.82 ±0.01^{c}	8.33 ±0.01^{a}	7.22 ±0.04^{b}	5.82 ±0.01^{d}
Overall impression	8.88 ±0.02^{a}	8.84 ±0.03^{a}	8.58 ±0.01^{ab}	8.87 ±0.04^{a}	8.66 ±0.01^{ab}	8.56 ±0.04^{ab}

Note: Values are means (n = 45) ± standard deviation. Means followed by different superscripts are significantly different (p <0.05) along column according to Duncan multiple range test: CPW, CPW-GIN, CPW-GAR, CPWGG2, CPW-ASC and CPW-SBZ represent juice that was unpreserved juice blend, preserved with 1% ginger, preserved with 1% garlic, preserved with 1% ginger-garlic, preserved with 1% ascorbic acid and preserved with 1% benzoate acid, respectively

Table 2 Variation in pH of the cashew, pineapple and water melon juice blends with the different preservatives during the period of storage.

Preservative type	Storage period (weeks)						% decrease
	0	1	2	3	4	5	
No added preservative	4.53 ±0.01^{a}	4.53 ±0.02^{a}	4.46 ±0.02^{a}	3.10 ±0.01^{c}	3.10 ±0.01^{b}	3.10 ±0.01^{b}	31.6
1% ginger	4.06 ±0.01^{b}	3.61 ±0.03^{d}	3.48 ±0.05^{d}	3.14 ±0.01^{c}	3.04 ±0.01c	3.04 ±0.01^{c}	25.1
1% garlic	4.51 ±0.02^{a}	4.38 ±0.01^{b}	4.28 ±0.02^{b}	3.42 ±0.01^{a}	3.13 ±0.03^{b}	3.12 ±0.01^{b}	30.8
1% garlic – ginger	4.47 ±0.04^{a}	4.43 ±0.01^{ab}	3.59 ±0.02^{d}	3.10 ±0.03^{c}	3.09 ±0.02^{b}	3.09 ±0.04^{b}	30.9
1% ascorbic acid	4.06 ±0.01^{b}	4.05 ±0.01^{c}	4.02 ±0.02^{c}	3.33 ±0.01^{b}	3.28 ±0.01^{a}	3.22 ±0.01^{a}	20.7
1% Sodium benzoate	4.03 ±0.02^{b}	4.02 ±0.04^{c}	3.52 ±0.02^{d}	3.06 ±0.04^{c}	3.02 ±0.03^{c}	3.02 ±0.01^{c}	25.1

Note: Values are means (n = 3) ± standard deviation. Means followed by different superscripts are significantly different (p <0.05) along column according to Duncan multiple range test.

Table 3 Variation in titratable acidity of the cashew, pineapple and water melon juice blends with the different preservatives during the period of storage.

Preservative type	Storage period (weeks)						% increase
	0	1	2	3	4	5	
No added preservative	0.11 ±0.02^{a}	0.17 ±0.01^{a}	0.17 ±0.02^{a}	0.19 ±0.01^{a}	0.21 ±0.01^{a}	0.21 ±0.01^{a}	90.9
1% ginger	0.09 ±0.01^{b}	0.09 ±0.01^{c}	0.17 ±0.01^{a}	0.17 ±0.03^{a}	0.17 ±0.02^{a}	0.21 ±0.00^{a}	133
1% garlic	0.05 ±0.01^{c}	0.09 ±0.02^{c}	0.13 ±0.01^{b}	0.13 ±0.01^{b}	0.13 ±0.02^{b}	0.17 ±0.01^{b}	240
1% garlic – ginger	0.14 ±0.01^{a}	0.14 ±0.00^{b}	0.20 ±0.01^{a}	0.20 ±0.01^{a}	0.19 ±0.01^{a}	0.19 ±0.01^{a}	35.7
1% ascorbic acid	0.07 ±0.02^{c}	0.12 ±0.01^{b}	0.14 ±0.00^{b}	0.18 ±0.02^{a}	0.18 ±0.02^{a}	0.21 ±0.01^{a}	200
1% Sodium benzoate	0.10 ±0.02^{a}	0.13 ±0.01^{b}	0.18 ±0.01^{a}	0.18 ±0.03^{a}	0.18 ±0.01^{a}	0.18 ±0.02^{a}	80

Note: Values are means (n = 3) ± standard deviation. Means followed by different superscripts are significantly different (p <0.05) along column according to Duncan multiple range test.

Table 4 Variation in specific gravity of the cashew, pineapple and water melon juice blends with the different preservatives during the period of storage.

Preservative type	Storage period (weeks)						% increase
	0	1	2	3	4	5	
No added preservative	1.32 ±0.04^{b}	1.38 ±0.01^{c}	1.70 ±0.00^{a}	1.75 ±0.00^{a}	1.85 ±0.01^{a}	1.91 ±0.00^{a}	44.7
1% ginger	1.55 ±0.01^{a}	1.57 ±0.01^{a}	1.61 ±0.01^{b}	1.63 ±0.01^{b}	1.65 ±0.02^{b}	1.68 ±0.02^{b}	8.4
1% garlic	1.56 ±0.00^{a}	1.57 ±0.00^{a}	1.58 ±0.03^{b}	1.61 ±0.03^{b}	1.63 ±0.03^{b}	1.63 ±0.02^{b}	4.5
1% garlic-ginger	1.49 ±0.03^{a}	1.51 ±0.04^{a}	1.52 ±0.02^{c}	1.54 ±0.02^{c}	1.56 ±0.01^{c}	1.58 ±0.05^{b}	6.0
1% ascorbic acid	1.37 ±0.01^{b}	1.37 ±0.02^{c}	1.54 ±0.01^{c}	1.57 ±0.02^{b}	1.59 ±0.00^{b}	1.62 ±0.03^{b}	18.2
1% Sodium benzoate	1.34 ±0.02^{b}	1.45 ±0.01^{b}	1.55 ±0.00^{c}	1.57 ±0.02^{b}	1.57 ±0.02^{b}	1.58 ±0.02^{b}	17.9

Note: Values are means (n = 3) ± standard deviation. Means followed by different superscripts are significantly different (p <0.05) along column according to Duncan multiple range test.

Table 5 Variation in total soluble solids of the cashew, pineapple and water melon juice blends with the different preservatives during the period of storage.

Preservative type	Storage period (weeks)						% increase
	0	1	2	3	4	5	
No added preservative	5.79 ±0.00^{c}	5.77 ±0.01^{b}	5.60 ±0.01^{c}	5.38 ±0.02^{d}	5.28 ±0.02^{d}	5.09 ±0.01^{d}	12.1
1% ginger	5.90 ±0.05^{a}	5.82 ±0.01^{b}	5.60 ±0.03^{c}	5.60 ±0.04^{b}	5.53 ±0.01^{b}	5.53 ±0.01^{b}	6.3
1% garlic	5.82 ±0.01^{b}	5.78 ±0.02^{b}	5.72 ±0.00^{b}	5.69 ±0.00^{b}	5.67 ±0.02^{a}	5.61 ±0.05^{a}	3.6
1% garlic-ginger	5.98 ±0.01^{a}	5.88 ±0.00^{a}	5.78 ±0.03^{a}	5.75 ±0.01^{a}	5.66 ±0.02^{a}	5.65 ±0.03^{a}	5.5
1% ascorbic acid	5.81 ±0.02^{b}	5.79 ±0.01^{b}	5.65 ±0.01^{c}	5.51 ±0.02^{c}	5.60 ±0.04^{a}	5.20 ±0.02^{c}	10.5
1% Sodium benzoate	5.83 ±0.02^{b}	5.80 ±0.01^{b}	5.74 ±0.00^{b}	5.71 ±0.02^{a}	5.45 ±0.02^{c}	5.28 ±0.01^{c}	9.4

Note: Values are means (n = 3) ± standard deviation. Means followed by different superscripts are significantly different (p <0.05) along column according to Duncan multiple range test.

Table 6 Microbial Counts of the Fruit Juice during Storage.

Samples	Storage period (weeks)				
	1	2	3	4	5
Total bacterial count (colony-forming units.mL^{-1})					
Unpreserved	80×10^{3}	25×10^{3}	43×10^{3}	30×10^{5}	10×10^{8}
Preserved 1% ginger	12×10^{3}	20×10^{3}	24×10^{3}	10×10^{4}	15×10^{5}
Preserved with 1% garlic	11×10^{3}	18×10^{3}	17×10^{3}	29×10^{3}	11×10^{4}
Preserved with 1% ginger-garlic	Nil	Nil	12×10^{3}	20×10^{3}	35×10^{3}
Preserved with 1% ascorbic acid	12×10^{3}	22×10^{3}	39×10^{3}	24×10^{3}	75×10^{5}
Preserved with 1% sodium benzoate	12×10^{3}	25×10^{3}	43×10^{3}	84×10^{4}	90×10^{5}
Lactic acid bacterial count (colony-forming units.mL^{-1})					
Unpreserved	36×10^{3}	39×10^{3}	54×10^{3}	25×10^{4}	25×10^{6}
Preserved 1% ginger	24×10^{2}	38×10^{2}	51×10^{2}	45×10^{3}	65×10^{3}
Preserved with 1% garlic	Nil	21×10^{2}	29×10^{2}	33×10^{2}	40×10^{3}
Preserved with 1% ginger-garlic	Nil	Nil	12×10^{2}	16×10^{2}	52×10^{2}
Preserved with 1% ascorbic acid	60×10^{2}	58×10^{2}	40×10^{3}	56×10^{3}	46×10^{4}
Preserved with 1% sodium benzoate	34×10^{2}	60×10^{2}	39×10^{3}	47×10^{3}	15×10^{4}
Total fungal count (spore-forming units.mL^{-1})					
Unpreserved	14×10^{3}	29×10^{3}	40×10^{3}	25×10^{5}	34×10^{6}
Preserved 1% ginger	Nil	15×10^{3}	33×10^{3}	42×10^{3}	15×10^{4}
Preserved with 1% garlic	Nil	Nil	25×10^{2}	10×10^{2}	60×10^{2}
Preserved with 1% ginger-garlic	Nil	Nil	Nil	Nil	11×10^{2}
Preserved with 1% ascorbic acid	10×10^{2}	31×10^{2}	46×10^{3}	30×10^{4}	35×10^{4}
Preserved with 1% sodium benzoate	15×10^{2}	43×10^{2}	20×10^{3}	18×10^{4}	30×10^{4}

With respect to sensory accetability, the fruit blend preseved with the 1% garlic-ginger blend was the most accepatble (p <0.05). In presence of the biopreseravtives, the phyicochemical patrameters of the fruit blend showed stability during storage.

The study concluded that ginger and garlic could be used as effective biopreservatives in fruit juice blends at a minimum concentration at 1% and recommended as potential replacement for the chemical preservative during storage juice blends from cashew water melon and pineapple. The outcome of this study may expand the utilization of ginger and garlic more often in fruit juice production, create more job opportunities and reduce seasonal losses and wastage of fruits like cashew.

CONCLUSION

From the findings of this study, the biopreservatives (1% ginger, 1% garlic and 1% garlic-ginger extracts) compared favourably with the chemical preservatives (1% sodium benzoate and ascobic acid) used for preservation of the cashew water melon and pineapple juice blend. In addition, the study revealed 1% garlic-ginger extract as the most effective biopreservatives of all the biopresevatives used.

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INVESTIGATION OF ZERANOL IN BEEF OF UKRAINIAN PRODUCTION AND ITS REDUCTION WITH VARIOUS TECHNOLOGICAL PROCESSING

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ABSTRACT

Synthetic growth stimulants are widely used to get high productivity of animals. These preparations can accumulate in the meat and their residual quantities will adversely affect the health of consumers. The purpose of the work was to monitor the content of zeranol, growth stimulant of ruminants in beef which goes to meat processing enterprises of the Western region of Ukraine and to determine the effect of heat treatment on its quantity. It was found out that 29.8% of beef samples taken at meat processing enterprises contained a stimulant for the growth of ruminant zeranol. It was found that during the storage of beef samples frozen at a temperature of -18 °C with different content of zeranol there is a decrease in its number. The most intense process of destruction of zeranol occurred during the first month of storage, during this period of time the amount of zeranol is reduced by an average of 20%, regardless of the initial content. Within two months of storage of frozen beef, the content of zeranol decreases by 28.2 ±0.17%, and at the end of the sixth month its quantity decreases to 33.2 ±0.58%. It was also found that the dynamics of zeranol reducing in beef samples with large quantities (22.5 $\mu g.kg^{-1}$) and small (2.3 $\mu g.kg^{-1}$) were the same. It was set up that during 30 min of meat cooking there was a decrease in the content of zeranol 24.7 ±0.23% and 32.0 ±0.35% for 60 min, compared to its content in fresh meat. At the same time, when stored in the frozen state and subsequent cooking, the reduction of zeranol content in meat was 39.3 ±0.3%. Therefore, it is proposed to revise and amendments into the regulatory documents of Ukraine regarding the control and supervision of the presence of hormone (zeranol) residues in meat and meat products in order to prevent their sale and consumption by humans.

Keywords: beef; zeranol; frozen meat; synthetic growth promoters; meat temperature processing

INTRODUCTION

The meat industry is one of the main branches of agriculture, which provides the population with food rich in high protein. Currently, beef is an important part of the human diet, since meat has good taste, high nutritional levels and is also considered a dietary product **(Tonu, 2013; Salata et al., 2017)**. However, various synthetic hormonal growth promoters have become widely used for short periods of time in animal husbandry, in particular: zeranol, trenbolone acetate, diethylstilbestrol and others **(Galbraith, 2002; Brynes, 2005; Azza, Sania and Weam, 2015; Lykholat, Grigoryuk and Lykholat, 2016)**. Meat obtained from animals on the use of anabolics is characterized by a gentle consistency and lower fat content. However, according to many researches of many scientists **(Larrea and Chirinos, 2007; Jeong et al., 2010; Wang et al., 2013)**, excessive amounts of residues of hormonal preparation in meat and meat products adversely affect the health of consumers, causing various metabolic disorders and causing cancer. Therefore, in the countries of the European Union it is forbidden to use hormonal preparations – stimulants of growth of live weight of ruminants and it is regulated by directives **(EC, 1996a; EC, 1996b)**. At the same time in countries of Latin America and the USA the use of synthetic preparations in animal husbandry is allowed by national legislation **(CFR, 1999)**.

Our attention was drawn to the synthetic stimulator anabolic zeranol, known as α-zearalanol, it is a non-steroidal estrogen of the lactone group of resorbic acid and is used as an implant in the ear of cattle to increase muscle mass **(Leffers et al., 2001)**. Ukrainian legislation does not oblige the definition of this anabolic in beef at slaughterhouses and when implemented within the state. Therefore, researches on the presence of zeranol in the bovine muscle grown in Ukraine in domestic scientific publications are virtually none.

Therefore, conducting experimental researches on the monitoring of zeranol in beef at meat processing enterprises in Ukraine will allow to determine the real status of its circulation. Besides, based on the obtained information, it will be possible to propose appropriate amendments to the legislation of Ukraine on the safety of meat and meat products.

The purpose of the work was to monitor the content of zeranol, growth stimulant of ruminants in beef which goes to meat processing enterprises of the Western region of Ukraine and to determine the effect of heat treatment on its quantity.

Scientific hypothesis

The main hypothesis of the investigation is in the detected beef containing the synthetic anabolic stimulant zeranol, which goes to meat processing enterprises of the Western region of Ukraine and the possible use of meat processing that would reduce the amount of meat.

MATERIAL AND METHODOLOGY

Research on beef meat was conducted during years 2016 – 2018. Samples of beef meat were selected at the meat processing enterprises of the Western region of Ukraine to determine the amount of zeranol. Determination of zeranol in meat was performed using the test system for enzyme immunoassay RIDASCRIN® Zeranol (Art. No.:R3301) (manufactured by firm Art-Biopharm/R-Biopharm, Darmstadt, Germany) at the Stepan Gzhytskyj Lviv National University of Veterinary Medicine and Biotechnologies.

Statistical analysis

Statistical processing of the results was carried out using methods of variation statistics using the program Statistica 9.0 (StatSoft Inc., USA). Non-parametric methods of research were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x) and the standard error of mean (SE) were determined. The difference between the comparable values was considered to be significant for $p < 0.05$.

RESULTS AND DISCUSSION

The results of the searches of the content of zeranol in fresh beef are shown in Figure 1. From Figure 1 it can be seen that, on average, 30% of beef samples taken from meat at processing enterprises in the Western region of Ukraine contained a stimulant for the growth of ruminant zeranols. The number of negative samples without zeranol content was 70.2 ±2.1%. The detected amount of zeranol in meat was different (Figure 2). The highest number of samples – 35.7% with the content of zeranol was found in the smallest range from 1 to 5 $\mu g.kg^{-1}$. The number of samples with zeranol content from 5.1 to 10.0 $\mu g.kg^{-1}$ was found in 28.6%. A significant number of samples – 21.5% contained zeranol at high concentrations from 10.1 to 15.0 $\mu g.kg^{-1}$ and 14.2% of the investigated samples had its content greater than 15.1 $\mu g.kg^{-1}$.

Thus, the conducted searches have established the fact of receipts at processing plants of the Western region of Ukraine of beef containing the prohibited in the European Union countries the stimulant of growth of live mass of ruminants – zeranol.

Considering that when consuming food products containing residues of hormonal preparation, there is a violation of metabolism in humans we conducted research of the influence of different technological processes of temperature meat processing on reducing of zeranol content. In Figure 3 the results of the research are presented of changes in the content of zeranol in beef during its storage in the frozen state at a temperature of -18 °C.

The results of the research indicate that there is no complete reduction in the content of zeranol in meat during the six-month refrigerated storage. However, we note the same dynamics of zeranol reducing in beef samples, which contained both large and small amounts. The most intense process of destruction of zeranol occurred during the first month of storage. During this period of time, the amount of zeranol is decreased by an average of 20% in all samples, regardless of initial content. During the two-month term of storage of frozen beef, the content of zeranol was decreased by 28.2 ±0.17%, compared to the initial amount in fresh meat. Next storage of beef for three months did not cause a significant decrease in the content of zeranol and at the end of the sixth month their number was decreased by 33.2 ±0.58%.

Analogical results of the research on the dynamics of changes in zeranol in beef were obtained using the freezing temperature of meat -25 °C and -30 °C. However, when storing meat containing zeranol in the cooled state at +2 to +4 °C and frozen for -2 to -3 °C for 20 days, no decrease in the amount of zeranol was observed.

Thus, obtained scientific data indicate that the storage of meat in the frozen state has a positive effect on the dynamics of reducing the content of zeranol. This process is particularly active within two months of the start of storage.

In Figure 4 results of researches are given of the influence of the cooking process on the dynamics of changes in zeranol in beef. Installed that the process of cooking also affects to reduce the content of zeranol in beef. After 30 minutes of meat cooking, the amount of zeranol in all samples was decreased by 24.7 ±0.23% and over the next 30 minutes to 32.0 ±0.35%, compared to its content in fresh meat. Further heat processing up to 120 minutes did not cause a significant decrease in the zeranol content compared to the 60 minutes of cooking process.

Consequently, during cooking, about 30% of the zeranol from the meat to the broth is destroyed or transferred, which is almost the same amount as in the frozen storage process.

Changes in the content of zeranol in meat were also examined after 6 months of storage in the frozen state and subsequent cooking for 60 min (Figure 5). After freezing, the decrease in the amount of zeranol was found to be 33.2 ±0.58%, and the subsequent meat cooking process led to a slight reduction of the zeranol content to 6.1 ±0.2%. Therefore, in general, after the freezing process and further meat cooking process, the amount of zeranol is reduced by 39.3 ±0.3%.

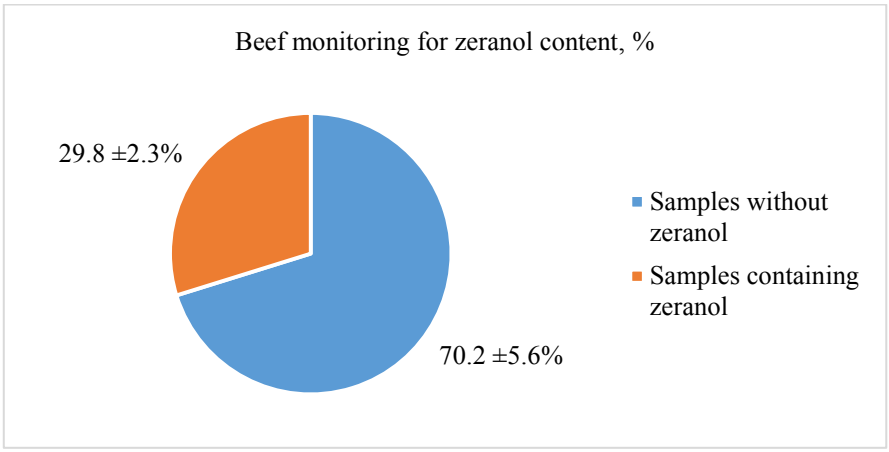

Figure 1 Searches of beef for the presence of zeranol.

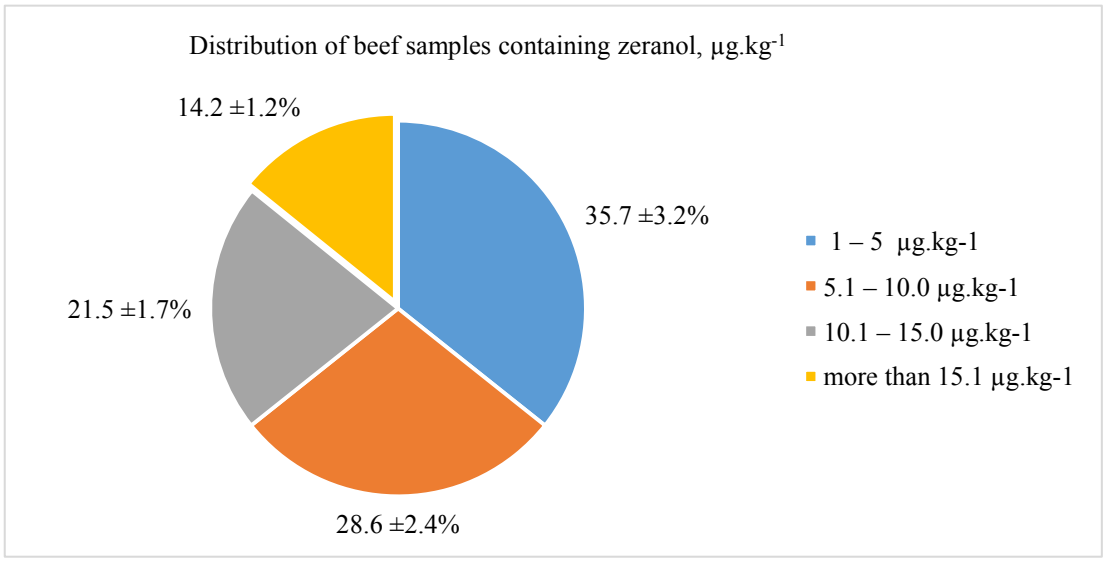

Figure 2 Characteristic of beef samples by zeranol content.

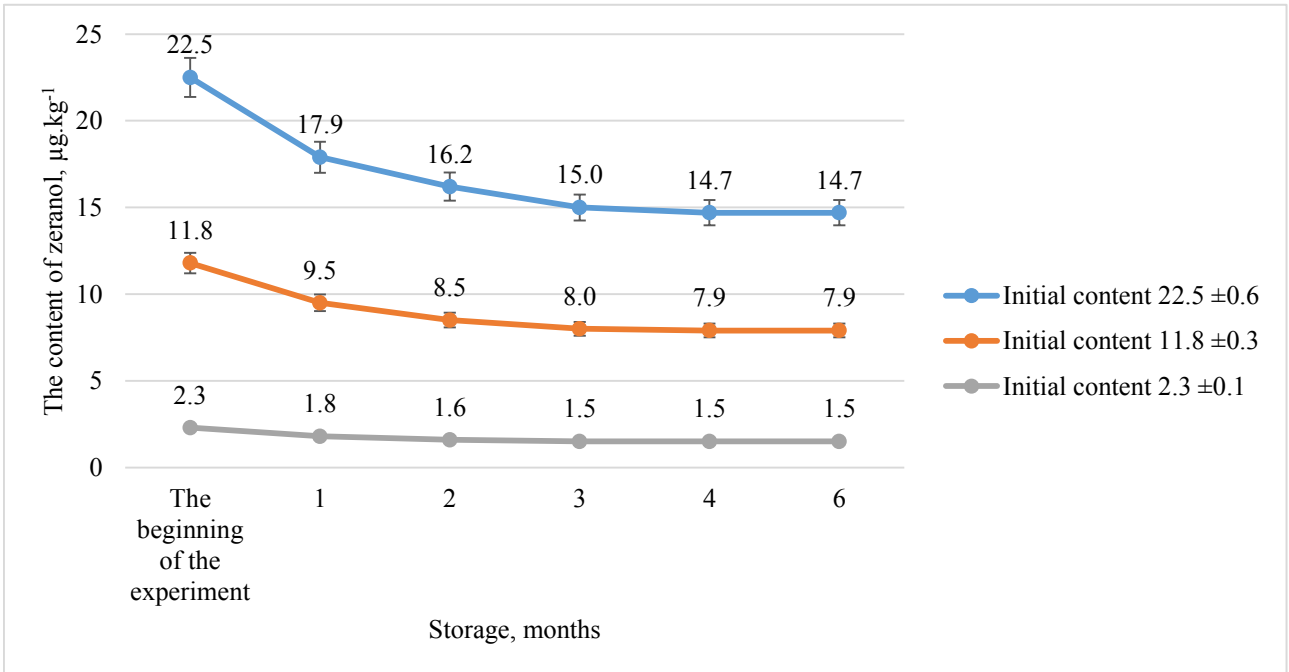

Figure 3 The intensity of the process of reducing zeranol in frozen beef for storage at a temperature of -18 ±1 °C for 6 months.

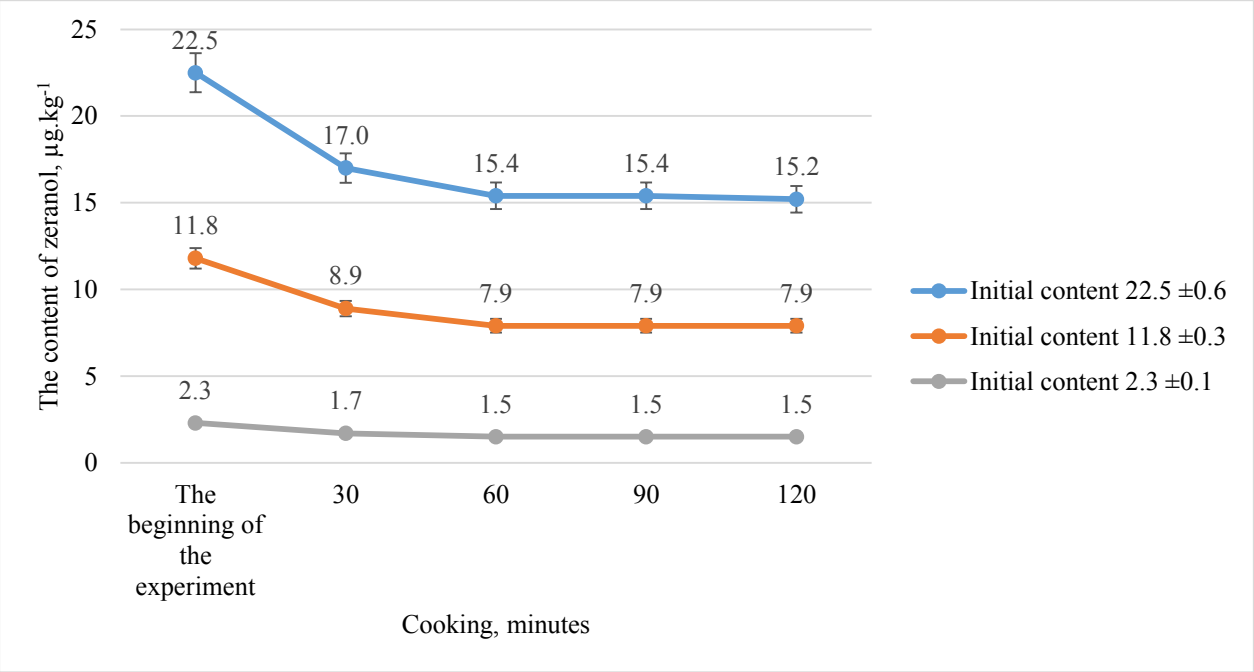

Figure 4 The intensity of the process of reducing zeranol in beef after cooking.

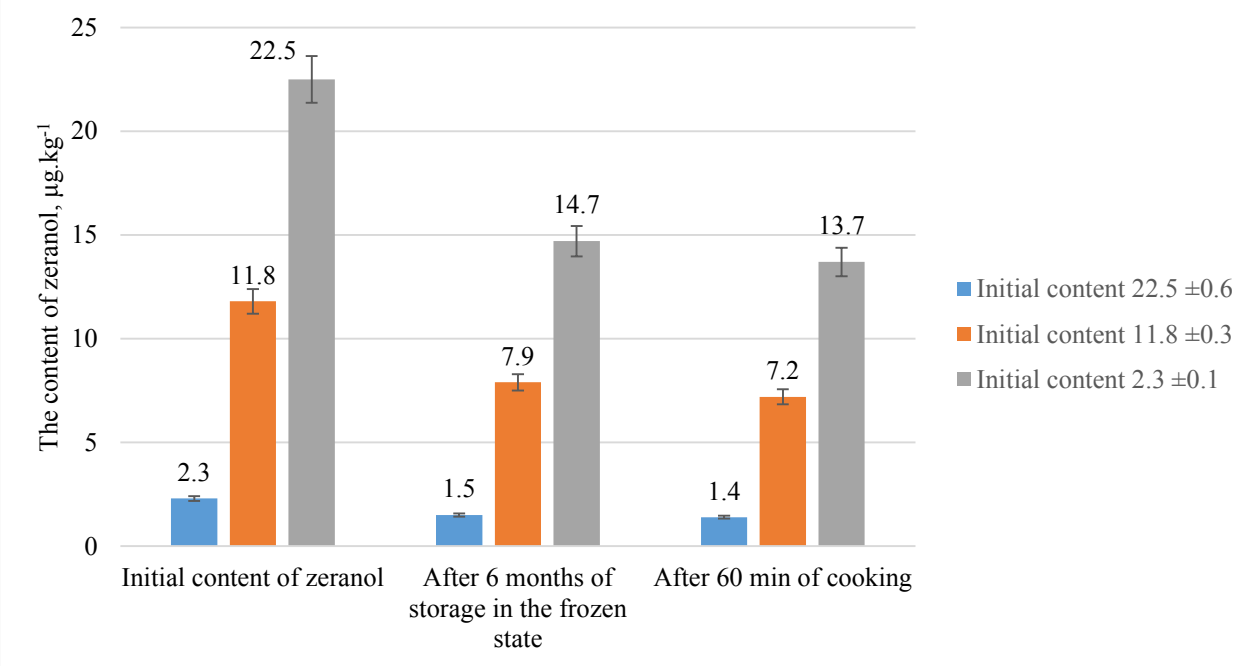

Figure 5 The intensity of the process of reducing zeranol in beef after 6 months of storage and 60 min of cooking

Meat and meat products are essential components of the human diet, so first of all they must be safe in biological, chemical and physical terms. It is currently widely used in the world to increase the growth of live weight of animals and improve the use of feed anabolic hormonal preparations of synthetic production **(Galbraith, 2002; Brynes, 2005; Azza, Sania and Weam, 2015; Lykholat, Grigoryuk and Lykholat, 2016)**. However, in violation of the fattening technology, these preparations can accumulate in the products of slaughter, and their residual quantities adversely affect the health of consumers **(Larrea and Chirinos, 2007; Jeong et al., 2010; Wang et al., 2013)**. Synthetic anabolic stimulator – zeranol is banned in the European Union for use in veterinary medicine because of its adverse influence on humans. At the same time in the USA this preparation is allowed and its amount in ruminant muscles is allowed up to 2 $\mu g.kg^{-1}$ and 10 $\mu g.kg^{-1}$ in liver **(CFR, 1999)**. In Ukraine, regulatory and legal documents do not regulate the definition of this anabolic in domestic beef and imported abroad.

Our research has found that 29.8% of beef samples taken at meat processing enterprises in the Western region of Ukraine contained a stimulant for the growth of ruminant

zeranol. In addition, a significant number of samples – 35.7% contained zeranol at high concentrations greater than 10 μg.kg^{-1}. Our researches are consistent with the results of scientists from other European countries **(Borazan et al., 2007; Salata, et al., 2017)**, who reported the detection of zeranol in 100% of the investigated meat samples and meat products. Consequently, conducted research have found that there is an income for the processing of beef, which contains the hormone to stimulate the growth of live weight – zeranol. Therefore, we consider it necessary to revise and making changes in the regulatory and legal documents of Ukraine on the control and supervision of the presence of residues of hormone preparations (zeranol) in meat and meat products in order to prevent their sale and consumption by humans. In addition, at the present time, due to the large number of food supplies from different countries, it is impossible to guarantee the safety of meat in terms of residues of animal growth stimulants **(Gladij and Sychevs'kyj, 2018)**. Therefore, when importing beef in Ukraine, it is necessary to control it for the presence of residual quantities of zeranol.

Considering the fact of receipts for beef processing with high content of zeranol, we conducted a research to determine the influence of different types of heat treatment and storage of beef on the quantitative content of zeranol. It has been established that storage of beef in chilled and frozen state for 20 days does not affect the content of zeranol. At the same time, it was found that the storage of beef samples in the frozen state at a temperature of -18 °C with different content of zeranol decreases its amount. Thus, the most intense process of destruction of zeranol occurred during the first month of storage, during this period of time the amount of zeranol was decreased by an average of 20%, regardless of the initial content. During the two-month term of storage of frozen beef, the content of zeranol was decreased by 28.2 ±0.17%, and at the end of the sixth month its amount was decreased to 33.2 ±0.58%. It was also found out that the dynamics of zeranol reducing in beef samples with large quantities (22.5 μg.kg^{-1}) and small (2.3 μg.kg^{-1}) were the same. Therefore, conducted researches have given us reason to affirm that the storage of meat in the frozen state has a positive influence on changes in the content of zeranol, that is, it significantly reduces its amount. With the small content of zeranol in meat, it can be reduced to the limit of 2 μg.kg^{-1} in the US. In addition, it was found out that during 30 min of cooking meat there is a decrease in the content of zeranol by 24.7 ±0.23%, and for 60 min by 32.0 ±0.35%, compared with its content in fresh meat. Further heat processing did not cause a significant decrease in the content of zeranol. At the same time, when stored in the frozen state and subsequent cooking, the reduction of zeranol content in meat was 39.3 ±0.3%. In researches **(Braekevelt et al., 2011)** it was also found a decrease in the amount of estrogen hormones in beef by 25 – 30% after its two-hour cooking. Moreover, the researchers found that in the non-fat beef, the process of disintegration of hormonal preparations was less and ranged from 5 to 20%. Thus, cooking reduces the content of anabiolics in meat, but in the presence of high concentrations, this process is not effective enough to produce a safe product.

CONCLUSION

In summary, it can be noted that the revealed fact of earnings to the processing of beef containing zeran in high concentrations, therefore, we consider it necessary to carry out a selective control and selection of beef at meat processing enterprises in order to establish safety indicators, namely the content of zeranol. To reduce the number of detected samples, the meat must be frozen at -18 °C and stored for at least two months and then subjected to heat processing (cooking). However, if significant concentrations of zeranol (greater than 2 μg.kg^{-1}) are found, such meat is prohibited. Consequently, the planned monitoring will allow the monitoring and analysis of the situation of beef zeranol in Ukraine.

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TECHNICAL EFFICIENCY AND FACTORS AFFECTING RICE PRODUCTION IN TIDAL LOWLANDS OF SOUTH SUMATRA PROVINCE INDONESIA

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ABSTRACT
Rice has been the staple food for most Indonesians, so the rice consumption in Indonesia is considerably high. Rice is cultivated in many agroecosystems, including tidal lowlands. Some tidal lowlands are considered suitable for rice cultivation. Therefore, tidal lowlands may support food security in Indonesia. However, productivity remains a problem in which inputs are not used efficiently. This study aims to determine the technical efficiency and identify factors affecting rice production in tidal lowlands of South Sumatra, one of main rice barns in Indonesia. A survey was conducted by interviewing 93 farmers in Telang Rejo Village. A data envelopment analysis (DEA) with output-oriented and variable returns to scale (VRS) approach was applied to measure technical efficiency score from each farm observed. An ordinary least square (OLS) regression with a Cobb-Douglass production function approach was employed to analyse the factors affecting rice production in tidal lowlands of South Sumatra, Indonesia. The results showed that majority of rice farms in the tidal lowlands of South Sumatra Indonesia were inefficient. There were 44 rice farms (47.31%) that were efficient, 5 rice farms (5.38%) that were inefficient under increasing returns to scale and 44 rice farms (47.31%) that were inefficient under decreasing returns to scale. The inputs, such as nitrogen, phosphorus, and potassium fertilisers, herbicides, insecticides and fungicides had positive significant influences on rice production in the tidal lowlands of South Sumatra, Indonesia.

Keywords: technical efficiency; rice production; tidal lowlands; data envelopment analysis; ordinary least square

INTRODUCTION
Rice is a staple food and livelihood platform for Asian countries, such as Vietnam, Indonesia, Pakistan and others **(Roy, Chan and Rainis, 2014; Roy, Chan and Xenarios, 2016; Al-Mashadani and Mahmood, 2019)** Rice is still an income source for rural society in Indonesia. Therefore, it has a strategic position. Moreover, Rice also contributes to 9.5% of the Gross Domestic Product of Indonesia **(Adriani and Wildayana, 2015; Central Bureau of Statistics of Indonesia, 2019)**. According to **Government regulation #12 of 2012** concerning food, the Indonesian government is obligated to guarantee availability, affordability, and fulfilment of food consumption for all Indonesians. However, the consumption rate of rice is very high in Indonesia. The rice consumption of Indonesia achieved approximately 29.13 million tons or 111.58 kg per capita per year **(Central Bureau of Statistics of Indonesia, 2017)**. A population rate increase caused rice consumption to also increase. At the same time, a high land conversion causes unstable rice production for fulfilling rice consumption in Indonesia. This means that the government must take an appropriate decision or policy for solving those problems.

One of the solutions taken by government is the development of agriculture in the suboptimal lands of Indonesia. One of the suboptimal lands in Indonesia is tidal lowland. Tidal lowland is reclaimed swamp land and occurs between the land and the sea. Therefore, tidal lowland depends on the changing tides. Tidal lowland has potential to support food security in Indonesia. One of the available tidal lowlands of Indonesia is in South Sumatra Province. Based on data, the total of tidal lowland area is 266,674 hectares in South Sumatra. A large number of tidal lowlands in South Sumatra are in Banyuasin Regency. Based on the report of statistics, the total of tidal lowlands in Banyuasin is 161,917 hectares **(Central Bureau of Statistics of Banyuasin Regency, 2018)**. Due to the available total area of tidal lowland is being large, it is expected become a food barn or food growing area or rice production centre of Indonesia. On the other hand, tidal lowland is still not reclaimed in large amounts because of peat in tidal lowlands. Therefore, Many tidal lowlands may not be utilised **(Susanto, 2003)**.

One of locations for tidal lowlands is in Telang Rejo Village, Delta Telang I, South Sumatra Province of Indonesia. This location is a reclamation project in the

1970s involving the transmigration program from Java Island to Sumatra Island **(Scholz, 1980; Wildayana, Adriani and Armanto, 2017; Wildayana and Armanto, 2018)**. Telang Rejo Village has type A tidal lowlands. The type A is a tidal lowlands suitable for rice cultivation **(Imanudin and Armanto, 2012)**, it forms by tidal lowlands that are overflowed both large and small tides at all times **(Irwandi, 2015)**.

Besides the good potential of tidal lowlands, the constraints and threats exist, such as the rice productivity of tidal lowlands still being low. The rice productivity of tidal lowlands is approximately 4.10 to 4.43 tons per hectare. Whereas, the average rice productivity is 8 tons per hectare **(Wildayana and Armanto, 2019)**. This difference between actual and expected production was caused by inefficient use of input **(Majumder et al., 2016)**. The other problems of rice cultivation in tidal lowlands are soil acidity, nutrient deficiency, salinity and pyrite (Fe_2S) content **(Armanto, et al., 2013; Armanto, 2014; Wildayana and Armanto, 2018)**. Furthermore, the water is unsuitable for the crop needs for rice cultivation in tidal lowlands agriculture **(Yazid et al., 2015)**. There is no technological recommendation based location, such as fertiliser, variety of rice, land clearing and management, and also water management **(Oemar, 2003)**. These factors are causes of inefficient use of agricultural inputs to rice production in tidal lowland agriculture.

Studies regarding the technical efficiency of rice production become important for estimating the efficiency level of rice production in tidal lowlands agriculture. Technical efficiency refers to achieving a total of potential output though s combination of total of available input **(Iráizoz, Rapún, and Zabaleta, 2003)**. If a business or enterprise can improve output by input use optimization, the business is efficient **(Coelli et al., 2005)**. Some studies regarding the technical efficiency of rice production in Indonesia have been conducted **(Erwidodo, 1990; Squires and Tabor, 1991; Trewin et al., 1995)**. Even through there have been many studies regarding the efficiency of rice production in Indonesia, there are only a little of them discussing the efficiency of rice production in tidal lowlands of South Sumatra in particular. These previous studies investigated phenomena on Java Island with technical irrigated land agroecosystem. On the other hand, this study investigates the case of Sumatra Island with its suboptimal land agroecosystem, which is tidal lowlands. Therefore, this study is very important to be conducted.

Scientific hypotheses

There were two hypotheses in this study:

1. 70% of rice farms in tidal lowlands of South Sumatra Indonesia are efficient in constant returns to scale condition
2. Land area, seed, fertilizer of N, P, and K fertilisers herbicides, insecticides, fungicides and labour significantly affect rice production in tidal lowlands of South Sumatra Indonesia

MATERIAL AND METHODOLOGY

Location

This study was conducted in Telang Rejo Village, Banyuasin Regency, South Sumatra Province of Indonesia. There are some considerations in choosing the location.

1. This location has tidal lowlands of type A, and this location is suitable for rice cultivation.
2. This location is well known as food barn and production centre of tidal lowland rice for Banyuasin Regency.
3. This location is the largest village in tidal lowland agriculture of South Sumatera.
4. Telang Rejo is one of the transmigration project villages in the 1970s. The tidal lowlands in Telang Rejo was reclaimed by government to transmigrants from Java Island to Sumatra Island.
5. The Water management system was built by the agency of public work through grants from the government. The map of study site is in Figure 1.

Data Collection

Data was obtained by interviewing 93 farmers in Telang Rejo. Therefore, there were 93 decision making units (DMUs). They were selected randomly. This study used a questionnaire as a tool of research. This study was assisted by five master degree students as enumerators. The variables used in this study consisted one dependent variable (Y) and nine independent variables (X). The dependent variable was the rice production of tidal lowlands **(Coelli et al., 2005)**. The independent variables were land area **(Thanh Nguyen, Hoang and Seo, 2012)**, seeds **(Duangbootsee and Myers, 2014)**, chemical fertilisers which are N, P and K **(Hoang and Alauddin, 2012; Hoang and Nguyen, 2013; Jansen et al., 2006)**, pesticides and labour **(Rios and Shively, 2005; (Duangbootsee and Myers, 2014)**.

Technical Efficiency

Efficiency is the ratio between output and input. If the ratio is high, the efficiency score will be high. The efficiency score is between 0 to 1. It can be defined mathematically $0 \leq TE \leq 1$. Efficiency score can be obtained by **(Arnade, 1994)**:

$$Efficiency = \frac{\sum_k u_k y_{k,j}}{\sum_i v_i x_{i,j}}$$

Where :
y = output
x = input
u, v = average weight
i,j,k = 1,2,3,...n

Data Envelopment Analysis (DEA)

Data envelopment analysis (DEA) is a method to estimate efficiency with linear programming nonparametric approach **(Charnes, Cooper and Rhodes 1978; Charnes, Cooper and Rhodes, 1979)** DEA identifies the best frontier solution involving all observation of decision making units (DMUs). Therefore, it is called an envelope model. The DEA model also can be applied to estimate efficiency or performance in some sectors such as hospital **(Gholami, Higón and Emrouznejad, 2015; Khushalani and Ozcan, 2017)**,

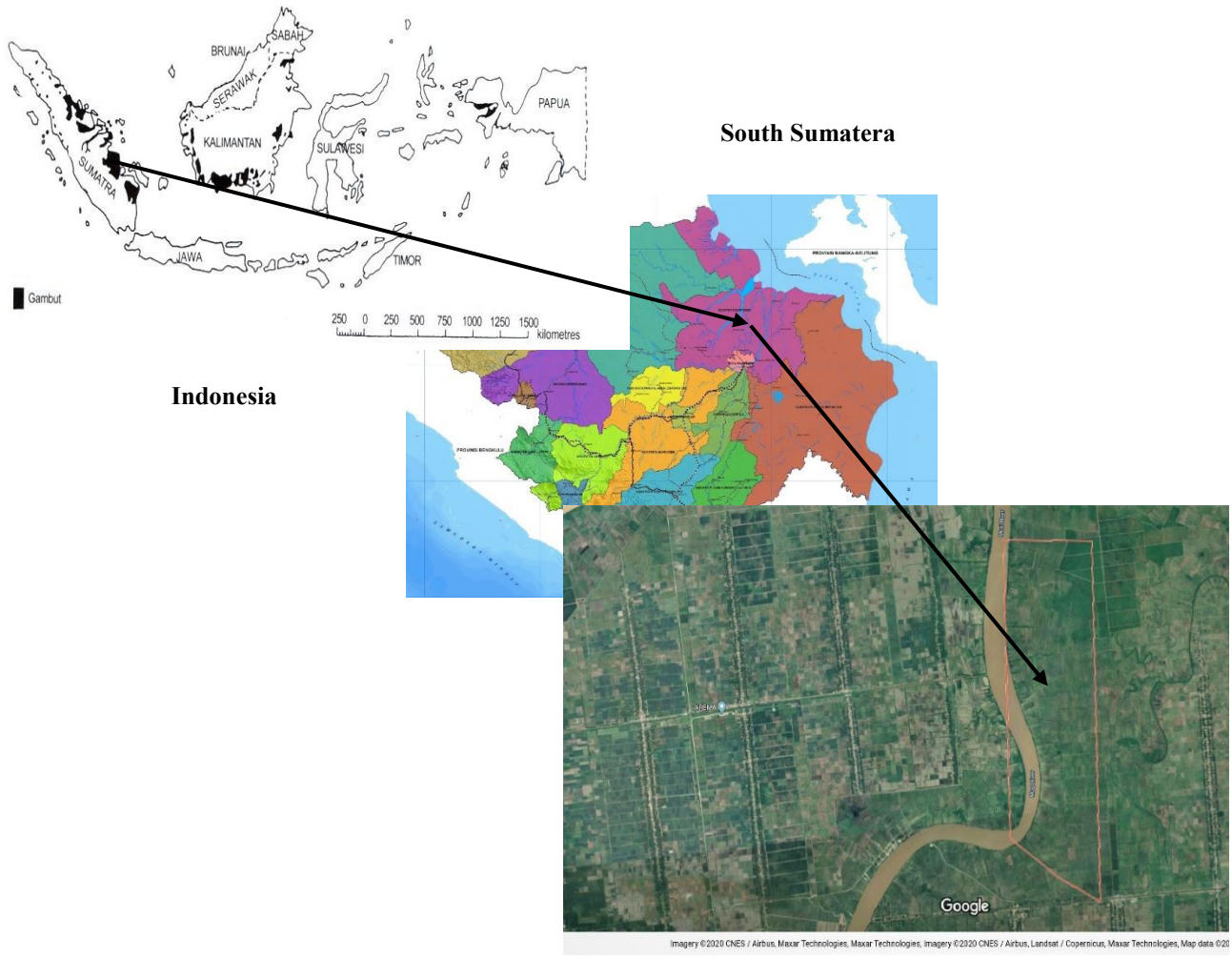

Figure 1 Location of study.

school **(Fatimah and Mahmudah, 2017)**, construction **(Hu and Liu, 2016)**, banking **(Wanke and Barros, 2014; Avkiran, 2015)**, port operation **(Rajasekar and Deo, 2014; Nguyen et al., 2016)**, industry and manufacture **(Kotey and O'Donnell, 2002)**, agriculture **(Haji, 2007; Guzman et al., 2009; Mardani and Salarpour, 2015)** and among others.

DEA has also been applied widely to estimate farm and agricultural performance worldwide **(Kočišová, 2015; Wang et al., 2017, Wang et al., 2018; Adeyonu et al., 2019)**. DEA has been used as a tool for decision making in a businesses, organisations, and governments as well. DEA has some advantages. It can be applied simultaneously by multiple inputs and ouputs. Without the need to previously determine weights. Furthermore, DEA does not need a functional form and specific production function such as input-output relationship.

DEA is divided by 2 orientations. The first is oriented input. It minimises input to achieve a potential output level. The second one is oriented output. It maximises output bundle while keeping the input level constant. Both of them minimise input and maximise output for achieving efficiency, DEA model oriented input focuses on operational and managerial problems while DEA model oriented output is in regards to planning and strategy **(Cullinane, Song and Wang, 2005)**.

The asssumptions of the DEA model are constant retun to scale (CRS) and variable returns to scale (VRS). These assumptions affect the envelope frontier. CRS is difined as a proportion of input addition equaling proportion of output addition. VRS is defined as production technology showing increasing, constant or decreasing returns to scale. The VRS assumption is appropriate for agriculture study because agricultural production may occur in 3 situations which are increasing, constant or decreasing returns to scale.

The DEA model in this study applied oriented output. The model can defined as **(Färe, Grosskopf and Lovell, 1985; Ali and Seiford, 1993; Färe et al., 2017; O'Donnell, 2018)**:

$$Max\ \theta i \qquad (1)$$

Subject to:

$$u_{jm} \leq \sum_{j=1}^{j} z_j u_{jm} \qquad (2)$$

Where:

θ = output efficiency of DMU's being estimated by DEA

u_{jm} = amount of output m produced by DMU j

z_j = intensity variable for DMU j

j,m = 1,2,3...n

The model obtained is a CCR or CRS model. To transform the model to be VRS or BCC Model, this constraint below should be add in the model.

$$\sum_j \lambda_j = 1 \qquad (3)$$

A DEA model with oriented output and VRS approach estimates technical efficiency though measuring potential output by 93 farmers or DMUs. The level of input (λ) is kept constant so that it obtains three stages of production, namely increasing, constant or decreasing returns to scale.

Ordinary Least Square (OLS)

The use of OLS has been widely applied by many scholars. OLS is amethod to identify the factors affecting agricultural production. OLS is also used as a second phase analysis of the DEA. According to **Banker, Natarjan and Zhang (2019)** the application of the combination between DEA and OLS is better than the Simar-Wilson model in measuring productivity at the second stage. The application of OLS is also more consistent when combined with DEA in second stage **(Simar and Wilson, 2007)**. The OLS equation used in this study is the Cobb-Douglass production function equation. We can define it as :

$$Y = f(X1, X2, X3 \ldots Xn) \qquad (4)$$

$$Y = \beta 0 X1^{\beta 1} X2^{\beta 2} X3^{\beta 3} \ldots Xn^{\beta} \qquad (5)$$

The estimation of the above OLS equation was transformed by natural logarithm (Ln). It was created to obtain the equation or model easier to interpret **(Koutsoyiannis, 2001)**.

$$LnY = Ln\beta_0 + \beta_1 LnX_1 + \beta_2 LnX_2 + \beta_3 LnX_3 \ldots \beta_n LnXn \qquad (6)$$

Where:

Y	= Production
$\beta 0$	= Intercept
$\beta_1, \beta_2, \beta_3 \ldots \beta n$	= Parameter
$X_1, X_2, X_3 \ldots Xn$	= Input
n	= 1,2,3....n

Statistical analysis

The software used for data analysis in the study was the Data Envelopment Analysis Program (DEAP) software version 2.1. The software was developed by Tim Coelli from the Centre of Efficiency and Productivity Analysis (CEPA) in the University of Queensland, Australia. In addition, the Statistical Package for Social Science (SPSS) version 23 was also used for OLS analysis. The *p*-values used in OLS analysis were $p < 0.01$; 0.05 and 0.10

RESULTS AND DISCUSSION

Characteristics of farmers

Based on Table 1, farmers were in productive age group. The majority of farmers aged 40 – 49 years. The household size was 2 people for the majority. A small percentage of respondents were elderly and whose wife or husband has passed away lived alone. These respondents normally had children who were married and lived nearby. A low percentage of respondents had two or more children. These respondents normally are middle aged with children who are still young and unmarried. However, the education of farmers is still relatively low. The length of education only 4 to 7 years, which is equivalent to primary school. The majority of farmers had 11 – 20 years of farming experience. This was because farmers in Telang Rejo Village are the second and third generation of transmigrants of the transmigration project from Java Island to Sumatra Island in the 1970s. The land status of farmers in Telang Rejo Village was mainly private ownership. This is because at the beginning of the transmigration program each household was given 2 hectares of reclaimed tidal lowlands by the government **(Arsyad, Saidi and Enrizal, 2014)**.

Table 2 showed agricultural input uses and output produced in tidal lowlands of Telang Rejo Village. According to The Ministry of Agriculture of Indonesia (2007) the uses of N, P and K fertilisers are 200 $kg.ha^{-1}$, 75 $kg.ha^{-1}$ and 50 $kg.ha^{-1}$ resepectively . In fact, the uses of N, P and K fertilisers in tidal lowlands were higher than the recommendation. This could be a threat for sustainability and environmental integrity in tidal lowlands with theses surpluses.

Characteristic of Tidal Lowlands

Tidal lowlands contain pyrite and peat. Pyrite was formed when the tidal lowlands were flowed by sea water in dry season. It will be dangerous to rice when it was oxidized. Furthermore, peat was cause of soil acidity **(Shamshuddin et al., 2004; Nurita and Ar-Riza, 2014)**.

The tidal lowlands' problem are pH, nutrient deficiency, high content of Fe and Al and uncontrolled water **(Purnomo et al., 2005)**. Water management in tidal lowlands was different with irrigation system. The government had built some primary and secondary canals with the sliding gate and flap gate. In the rice farm, The intensive shallow canals were conducted which function to wash acidic and toxic substances from the field **(Widjaja-Adi, Ratmini and Swastika, 1997)**. However, the maintenance of the canals need to consider because some gates were broken **(Ar-Riza and Alkasuma, 2008)**.

Identification of efficient and inefficient rice farms in tidal lowland

A total of 93 farms were used for each efficiency score using the DEA method. The result of DEA showed that most of the tidal lowlands rice farms in Telang Rejo Village were inefficient. There were only 44 (47.31%) efficient rice farms that were in constant returns to scale condition, while there were 49 inefficient farms. Where 44 (47.31%) of those in decreasing returns to scale condition and 5 (5.38%) that were in in increasing returns to scale condition. Figure 2 is presented to show the results of the DEA.

There were 44 rice farms that had decreasing returns to scale caused by the excesses of input use. Therefore, to achieve efficiency the input uses need to be reduced. The purpose of reducing input use on the farms for the law of diminishing returns has not occurred. Whereas the farms with increasing returns to scale (five rice farms) should increase the input uses to achieve efficiency because it is still possible for them to increase production. The finding of a study stated that rice farms in Vietnam operated with less than the optimal scale **(Linh, 2012)**. The result supported this study.

Table 1 Descriptive statistics of farmers in Telang Rejo Village (n = 93).

Characteristics	Frequency	Percentage (%)
Age (year)		
20 – 29	12	12.90
30 – 39	23	24.73
40 – 49	36	38.71
50 – 59	18	19.35
60 – 69	4	4.30
Household size (individual)		
1	20	21.51
2	34	36.56
3	30	32.26
4	8	8.60
5	1	1.08
Education (year)		
0 – 3	3	3.23
4 – 7	52	55.91
8 – 11	17	18.28
12 – 15	20	21.51
≥16	1	1.08
Farming experience (year)		
0 – 10	18	19.35
11 – 20	38	40.86
21 – 30	18	19.35
31 – 40	18	19.35
41 – 50	1	1.08
Land Status		
Renter	4	4.30
Owner	80	86.02
Renter and Owner	9	9.68

Table 2 Descriptive statistics of input and ouput (n=93)

Variable	Mean	Std. Deviation	Min.	Max.
Production (kg.ha^{-1})	6,602.15	142.66	3,000.00	10,000.00
Land area cultivated (ha)	4.83	3.52	0.50	20.00
Seeds (kg.ha^{-1})	86.40	18.71	50.00	120.00
N fertiliser (kg.ha^{-1})	329.57	1.61	50.00	650.00
P fertiliser (kg.ha^{-1})	223.66	1.14	50,00	500.00
K fertiliser (kg.ha^{-1})	195.16	1.09	50.00	400.00
Herbicide (L.ha^{-1})	6.90	3.59	2.00	20.00
Insekticide (L.ha^{-1})	6.41	3.83	1.00	17.00
Fungicide (L.ha^{-1})	5.99	3.22	1.00	17.00
Labour (day.ha^{-1})	3.78	3.36	1.00	14.00

Farm performance can also be divided based on the efficiency score. **Jalilov et al. (2019)** divided 3 performance using efficiency scores based on: 1) the best performance which has an efficiency score between 0.90 to 1.00 or 90% to 100%. Farming in this category was called efficient farming 2) good performance, or farming that has an efficiency score of 0.80 to 0.89 or 80% to 89% also included in the efficient category, while for 3) poor performance is farming that has efficiency score under 0.79 or 79%. Farming with this score is categorised as inefficient.

Based on this grouping, there were 68 (73.12%) farms that had efficiency scores from 90% to 100%. Then, there were 17 (18.28%) farms that had efficiency scores between 80% and 89%. Meanwhile, 8 (8.60%) farms had score of less than 79%. Figure 3 was given to show the categorisation

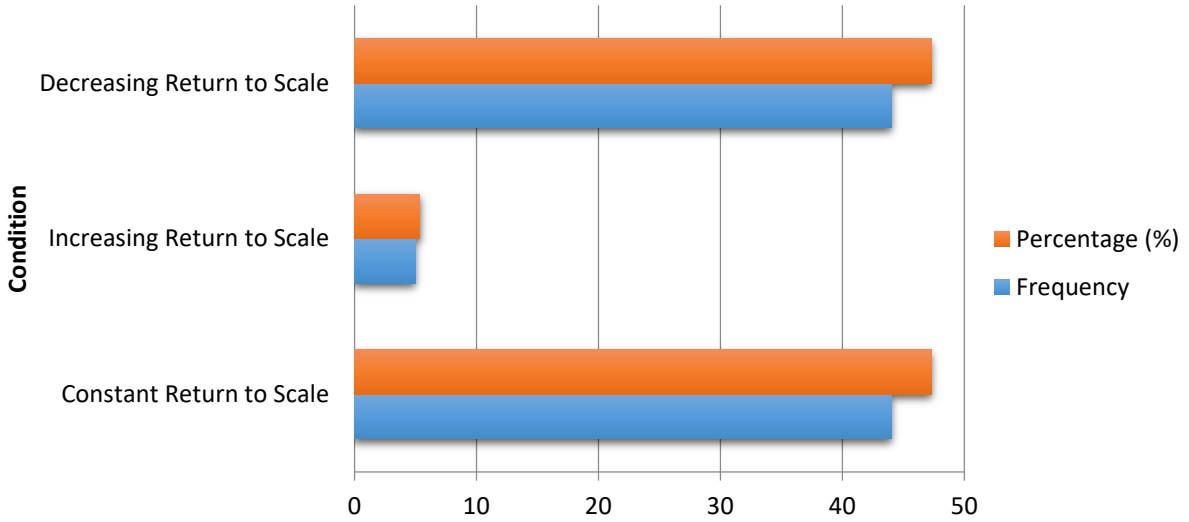

Figure 2 Distribution of farming based on returns to scale condition.

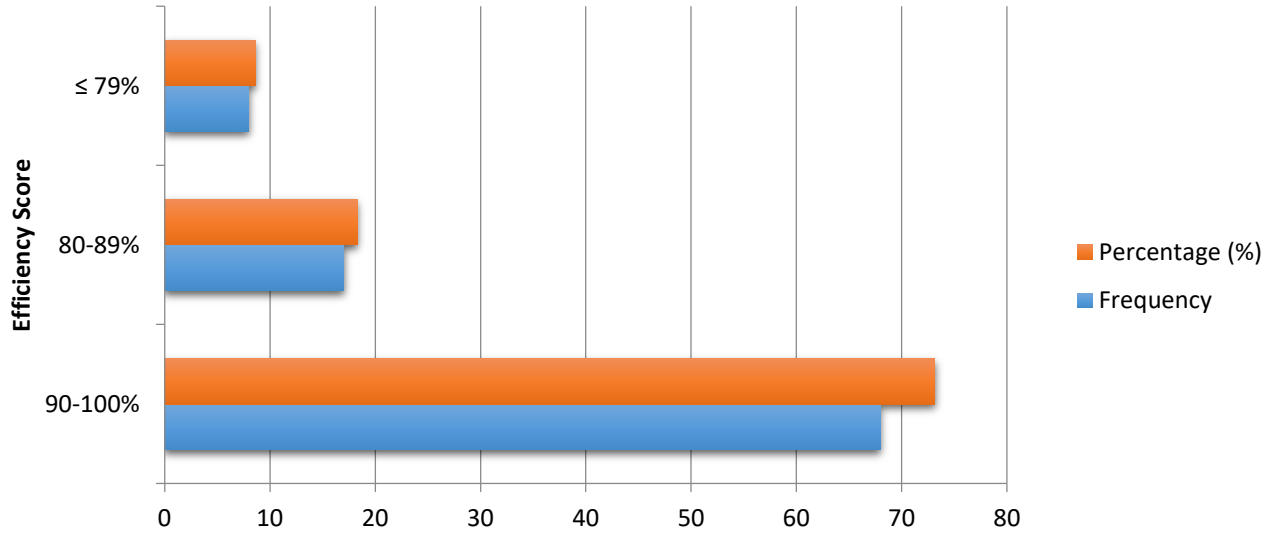

Figure 3 Categorisation of rice farming performance based on efficiency scores.

of rice farming performance based on efficiency scores. This finding is same like a study in Turkey. The average efficiency score of rice production in Marmara region was 92%. It means performance of rice farming in Turkey was the best performance **(Tipi et al., 2009)**.

The poorly performing farms occurred due to the input use not being optimal. The achievement of actual total production was not in accordance with the expected total production. It was further determined that poorly

performing farms were on decreasing returns to scale. The well performing farms with efficiency scores of 80% to 89% had worked on optimal input use. Furthermore, the best performing farms with efficiency scores of 90% to 100% had been on the efficiency frontier **(Jalilov et al., 2019)**.

Factors affecting rice production in tidal lowlands

This study applied an OLS analysis as the second stage analysis of DEA. The classic assumption test (data normality, heteroscedasticity and multicollinearity) was applied in OLS model. It was applied to obtain a free errors OLS model. In addition to obtain factors affecting rice production in tidal lowlands, OLS can also obtain an equation of the Cobb-Douglass production function for rice production in tidal lowland. The production functions obtained from OLS analysis were:

$$Y=127938130X1^{0.023}X2^{0.030}X3^{0.049}X4^{0.072}X5^{0.057}X6^{0.097}X7^{0.039}X8^{0.082}X9^{0.003} \quad (7)$$

Where:

Y = Tidal lowland rice production (kg.ha^{-1})
X1 = Land area cultivated (ha)
X2 = Seed (kg.ha^{-1})
X3 = N fertiliser (kg.ha^{-1})
X4 = P fertiliser (kg.ha^{-1})
X5 = K fertiliser (kg.ha^{-1})
X6 = Herbicide (L.ha^{-1})
X7 = Insecticide (L.ha^{-1})
X8 = Fungicide (L.ha^{-1})
X9 = Labour (day.ha^{-1})

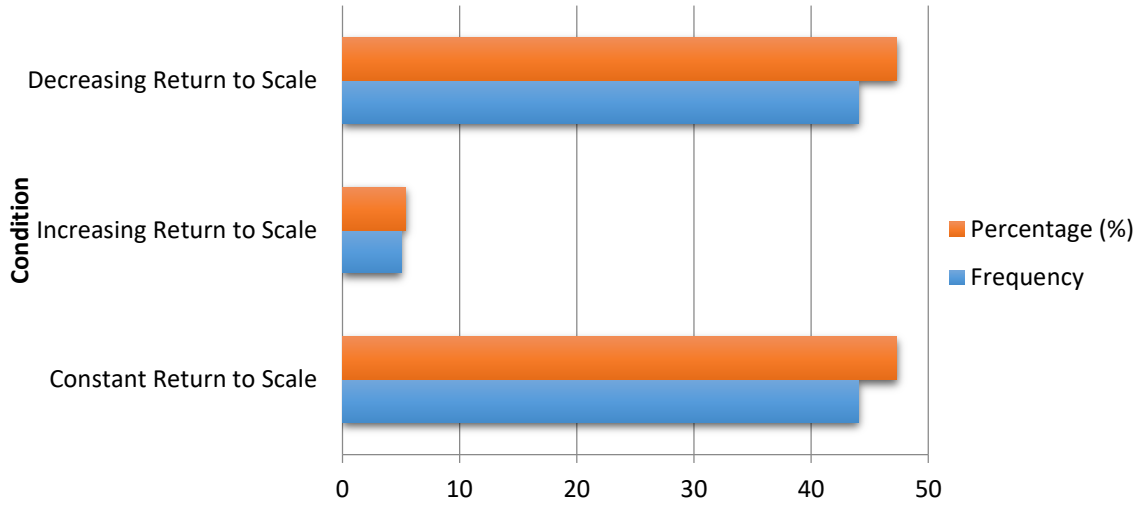

Figure 2 Distribution of farming based on returns to scale condition.

Table 3 The result of OLS analysis.

Variable	β	Std. Eror	t-stat	*p*
Intercept	8.107	0.274	29.549	0.000
Land area cultivated (X1)	0.023	0.027	0.862	0.391
Seed (X2)	0.030	0.061	0.498	0.620
Fertilizer N (X3)	0.049	0.026	1.885***	0.063
Fertilizer P (X4)	0.072	0.029	2.463**	0.016
Fertilizer K (X5)	0.057	0.032	1.794***	0.076
Herbicide (X6)	0.097	0.031	3.159*	0.002
Insectiside (X7)	0.039	0.022	1.809***	0.074
Fungicide (X8)	0.082	0.032	2.549**	0.013
Labour (X9)	0.003	0.022	0.135	0.893

Note: F-stat = 30.682; R-Square = 0.769; Adjusted R-Square = 0.744; * Significant at p <0.01; ** Significant at p <0.05; *** Significant at p <0.10.

Based on the OLS analysis (Table 3), it was found that herbicides had a positive significant influence on rice production in tidal lowlands (p <0.01), and P fertiliser and fungicides had a positive significant influence on rice production in tidal lowlands (p <0.05).

Furthermore, N Fertiliser , K Fertiliser and insecticides also had a significant influence on rice production in tidal lowlands (p <0.10). The findings supported a study by **Piya, Kiminami and Yagi (2012)**. The result of study stated that chemical fertilizer, pesticide and fungicide were positive and statistically significant to rice production in Nepal. Furthermore, pesticide and herbicide also affected rice production in Sri Lanka **(Gedara et al., 2012)**. Meanwhile, the land area cultivated, seeds and labour did not significantly influence rice production in tidal lowlands. A study stated that farm size did not affect rice production in Nigeria **(Ahmaedu and Alufohai, 2012)**. Many the land used by farmers in Telang Rejo Village were fragmented. The impact of fragmented land was that farmers had difficulty in managing rice farms. This was caused by the scattered distribution of land. Therefore, the management of rice farms in tidal lowland became ineffective. This case began when many farmers took a credit or loan from a wealthier farmer or *toke*. The land became a guarantee in this loan system. It was an informal credit with high interest. The interest applied by the *toke* is approximately 30% – 40% per year in a term of one to five years. The informal credit is absolutely not profitable for farmers in tidal lowland area. The loan system had terms and conditions as agreement between farmer and the *toke*. The farmers were not allowed to cultivate rice in their lands until they were able to pay their loan during the repayment period. The farmers who were not able to pay the loan according to agreement had to give their land to the *toke*. This situation created rich farmers with large land areas and poor farmers with small land area, so there was significant inequality among the farmers in tidal lowlands.

Seed did not significantly affect rice production in tidal lowlands. The use of seeds was very high in the rice farms of tidal lowlands. This finding was supported by **Dhungana, Nuthall and Nartea (2004)** and **Linn and Maenhout (2019)** . They stated that use of seed was very high in rice farming in Nepal and Myanmar. The use of excess seeds caused many farms to be in decreasing returns to scale condition. The use of excess seeds was mostly encouraged by cultivating system in tidal lowland. *Tabela* is well-known seedling system by farmers. In fact, the optimal use of seeds was 20 kg to 30 kg per hectare

with seeding. *Tabe*la is abbreviation from three words (*Tebar Benih Langsung*) in Bahasa Indonesia. *Tabela* was done by spreading seed out directly to land without first seeding. When *tabela* was applied, seeds would flow out with water. Therefore, seeds were useless. *Tabela* is often known as *sonor*. *Tabela* was followed by burning land in land preparation **(Wildayana, Armanto and Junedi, 2017)**. This cultivation system was a local wisdom for tidal lowland farmers in Telang Rejo Village. The cultivation system made spacing of crops irregular, and caused the rice production of tidal lowlands to be low. Furthermore, the varieties used by farmers are ciherang, IR 42 or others. That was developed by farmers with technology limitations. In addition, it is possibly not water stress tolerant variety. One of the problems in tidal lowlands is a need for water because tidal lowlands depend on tides. Therefore, a water stress tolerant variety is needed by farmers in tidal lowland agriculture to increase rice production. Inpara-3 is a suitable variety to tidal lowlands **(Saidi et al., 2014)**. However, the farmers do not adopt it yet.

Labour was also a factor that did not affect rice production in tidal lowlands. The available labour force in tidal lowland agriculture is very low. In fact, many transmigrant farmers sold their land and farms given by the government to return to their homeland on Java Island. Then, many farmers migrated to find other work in the capital city of South Sumatra Province (Palembang). It occurred along cultivation season. Therefore, a large labour force for agriculture and farming in Telang Rejo Village was not available. Moreover, some farmers changed their jobs from agricultural jobs to non-agricultural jobs. The majority of them worked in construction in the capital city of South Sumatra Province or became workers on an engine boat in the Musi River. Musi River is the longest river of South Sumatra. It is also used as transportation like Mekong River in Vietnam. The case of labour migration also occurred in China. The available labour for agricultural jobs has decreased significantly **(Peng, Tang and Zou, 2009)**. It will be a challenges of rice production. The other cause was agricultural mechanisation. Many farmers used machines for land preparation, harvesting and other activities.

CONCLUSION

This paper concluded that rice production on tidal lowlands was inefficient. Only 47.31% of rice farming was efficient. They were in constant returns to scale. Meanwhile, 5.38% of rice farming was inefficient under increasing returns to scale and 47.31% of rice farming was inefficient under decreasing returns to scale. The factors affecting rice production in tidal lowlands were N, P, and K fertiliser, herbicides, insecticides and fungicides. In terms of policy implications, the need for rice varieties tolerant to tidal lowlands, use of organic fertilizer such as livestock dung, compost and others to achieve sustainability of tidal lowlands, seed nursery training by agricultural extension and also policies regarding the use of agricultural inputs, including doses and the other factors, so that rice production in tidal lowlands can be improved to achieve efficiency and food security in Indonesia.

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NUTRITIONAL AND BIOLOGICAL VALUE OF PORK OBTAINED FROM ANIMALS FED WITH LYSINE AND METHIONINE

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ABSTRACT
The article presents material on the effect of the amino acids lysine and methionine in pig diets. Studies have been carried out to increase the live weight of animals, depending on the content of these amino acids in the diet when fattening up to 100 and 120 kg. Presented research results obtained during testing in certified and accredited laboratories. When growing gilts to live weight from 30 to 50 kg, a deficiency in the level of lysine was found, which reaches up to 29.6%, and methionine with cystine up to 23.1%. When growing animals from 81 to 120 kg of live weight, the lysine deficiency is 14.9%, and methionine with cystine is 8,8%. This necessitated balancing the diet of animals to the required level of amino acid content. The most effective on the growth and development of animals, as well as on indicators of pork quality, was the introduction of feed lysine in combination with methionine in pig diets.

Keywords: lysine; methionine with cystine; gilts; live weight; rations

INTRODUCTION
At present, the pig-breeding industry in the Russian Federation is developing quite dynamically and domestic pork production reaches more than 95% of the volume consumed, which provides the population of the country per person 26 kg of this type of product. However, in countries of world pork exporters in the USA, England, Germany, Canada, Denmark, Italy, Spain, more than 100 kg of meat is produced per person. Therefore, in Russia there is a need to further increase the production of pig meat, including not only through the use of breeding and genetic innovations, but also to improve animal breeding by improving the quality of feeding rations **(Mysik, 2008; Strekozov and Chinarov, 2012)**. Full and balanced nutrition of pigs in all nutrients is the key not only to a steady increase in meat productivity of animals, but also their reproductive qualities, as well as physiological development at all stages of growth **(Kukushkin and Filatov, 2011; Belous et al., 2018; Kulintsev, 2011; Nikonkov et al., 2015; Kumar et al., 2012; Smith et al., 1999)**.

The aim of the research was to study the effect of diets with a low protein level in pig diets, which is typical for the southern regions of our country, on the physiological parameters of animals, as well as on fattening and meat qualities of young animals.

Scientific hypothesis
Supplementation of lysine and methionine in pig diet will increase production of pork. In connection with the foregoing, it is advisable to conduct research aimed at studying the effect of the introduction of the amino acids lysine and methionine with cystine on the physiological development of animals, growth, development of young pigs, as well as on increasing production of pork.

MATERIAL AND METHODOLOGY
The studies were conducted at the Lenin collective farm breeding farm in the Surovikinsky district of the Volgograd region in the period from 2017 to 2019. For testing, 3 groups of purebred gilts of large white breed, 16 animals each, obtained as a result of the second farrow, were selected. Gilts were selected according to the principle of analogue pairs, considering gender, age, and physiological state. For the experiment, the feeding process was divided into three periods. The first period is from 30 to 50 kg of live weight; the second period is from 51 – 80 kg and the third period are from 81 to 120 kg of live weight. Control slaughter of experimental animals was carried out when 100 and 120 kg of live weight were achieved.

The animals were grown according to the zootechnical standarts adopted in Russia. According to the recommendations of the all-russian institute of animal

husbandry, the balance sheet experience was divided into preparatory and main periods. The preparatory period was carried out for 21 days, animals of all experimental groups received the main diet. The study of the balance of the diets of feeding experimental animals was carried out on the basis of the analysis of the actual diet by the content of protein, lysine, methionine with cystine in it at the Volgograd Regional Veterinary Laboratory, a comprehensive analytical laboratory of the State Scientific Research Institute of Nuclear Medicine and Pediatrics, and Volgograd. The analysis of the chemical composition of feed and animal metabolic products was carried out according to generally accepted methods of zootechnical analysis **(Alikaev et al., 1967; Zlebedev and Usovich, 1976)**. In all experimental groups, animals were fed using concentrated feeds (concentrate type of feeding). The first group received the main diet, while the content of digestible protein fluctuated in weighted periods of 119 g, 106 g and 98 g, respectively. To adjust the balance of diets for essential amino acids, they were carried out using a mixture of peas and meat and bone meal, and the lack of carotene in the diet was ensured by the introduction of alfalfa flour. Gilts of the second and third groups received the main diet. At the same time, animals of the second group to compensate for the lack of lysine received feed lysine, which was prepared according to GOST R 56913-2016, and in the third group, the lack of lysine and methionine was compensated by feed lysine with the addition of methionine. Additionally, chalk and sodium chloride were added to the diets of animals of all experimental groups to provide them with calcium, sodium and chlorine. General economic rations for the periods of experiment consisted of 98.2 concentrated feed and 1.8% roughage in the first period; in the second period - concentrated feed – by 98.6% and roughage – by 1.4%, in the third period – concentrated feed – by 98.6% and roughage – by 1.4%. The analysis of the content in the meat samples of experimental animals was studied on the basis of data obtained on an amino acid analyzer model (L-8800, "Hitachi", Ltd). The content of amino acids and minerals is shown in Figure 1.

Analyzing the data presented in Figure 1, we can draw the following conclusion: in the first period of the experiment, the deficiency of the diets of experimental gilts for digestible protein is 19.0%, lysine – 34.9%, methionine with cystine – 37,8%; for the second period – by 14.4; 29.6; 23.1%, respectively; in the third period, there was a deficiency in the amino acid lysine – 14.9%, methionine with cystine – 8.8%. The experimental gilts were kept separately in groups, feeding was carried out twice. The necessary amino acids (in dry form) were introduced into the feed mixture stepwise after thorough mixing in the diets of experimental gilts. A study of the growth and development of gilts was established on the basis of taking into account the monthly increase in live weight. Before the control slaughter of the experimental animals, weighing was carried out after 24 hours of fasting. Assessment of meat productivity of animal carcasses was studied in accordance with the "All-Russian Institute of animal Husbandry Methodological Recommendations for the assessment of meat productivity, quality of meat and subcutaneous fat of pigs". Sampling of the longest muscle of the back and adipose tissue was

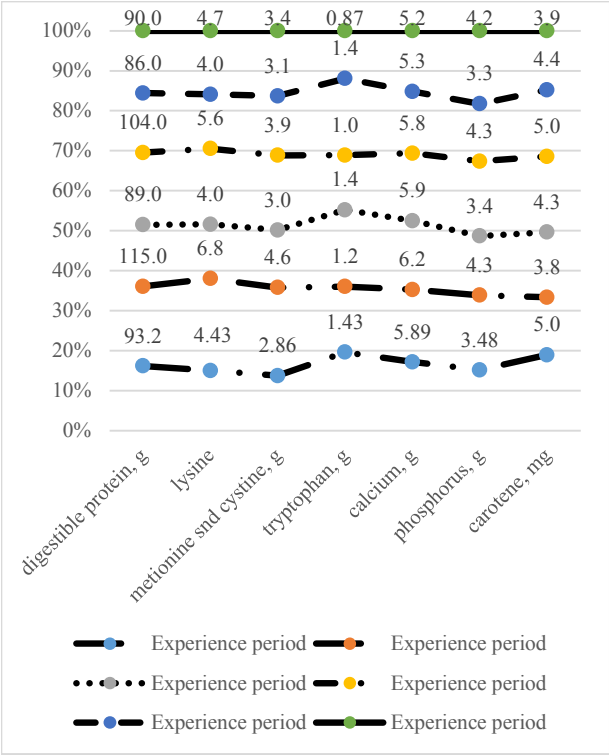

Figure 1 Diets of experimental animals.

carried out at a level between the 9th and 12th thoracic vertebrae, after cooling at a temperature of 2 – 4 °C in the refrigerator for 24 hours.

In order to more fully evaluate the meat qualities of experimental gilts after 24-hour exposure, the right half carcasses were deboned. At the same time, the weight of the ham, the ratio of meat, fat and bones, the area of the "muscle eye", determined on the cross section of the longest muscle of the back between the last pectoral and first lumbar vertebrae, were evaluated. Tasting evaluation of the broth and meat obtained from experimental animals was evaluated on the basis of organoleptic evaluation according to GOST 9959-91 "Meat products. General conditions for organoleptic assessment".

Statistic analysis

The data obtained during the experiments were processed using mathematical methods of variation statistics using the Microsof Excel software and Statistika 6 developer: StatSoft, USA. In the course of our work, we used the Student-Fisher method. For to assess the effect of lysine and methionine content in animal diets during different periods of their growth. The first threshold of reliability of the obtained data is designated as ($p \geq 0.95$); the second ($p \geq 0.99$); the third ($p \geq 0.999$), and if the results are not reliable, then ($p \leq 0.95$).

RESULTS AND DISCUSSION

The results of the dynamics of the live weight of experimental gilts are presented in Table 1. As we can see from the data in Table 1, animals of the 1st group were superior to analogues from groups 2 and 3 in live weight at 4, 5 and 6 months by 6.21 ($p \geq 0.95$) and 2.22%; 5.62 ($p \geq 0.95$) and 2.65% and 6,41 ($p \geq 0.95$) and 1.19%, respectively. However, when fattening up to 100 kg of live weight, the highest weight was achieved in group 2, which

is 0.60 and 0.20% higher in comparison with groups 1 and 3. Upon reaching 120 kg, the highest weight was observed in animals of the 3rd group, which is 0.33 and 0.83% more, respectively, in comparison with the 1st and 2nd groups. Based on the results of monthly weighings, the average daily gain in live weight was calculated, the data are presented in Figure 2.

As we can see from the data of Figure 2 and Figure 3, despite the fact that the average daily gain in live weight in animals of groups 2 and 3 at 5 months of age when reaching 100 and 120 kg of live weight was lower than in analogues of group 1 – by 97 and 40 g; 37 and 27 g and 28 and 13 g, the age of reaching 100 and 120 kg was less when reaching 100 kg of live weight by 9 and 5 days, when reaching 120 kg, animals of the 2nd group exceeded the analogues of the 1st group by 11 days, and analogues of 3 groups spent less by 24 days. It should be noted that the balance of plant general rations allows to significantly ($p \geq 0.95$) reduce feed costs (Figure 4). From the presented Figure 4 it can be seen that in animals of groups 2 and 3, when fattening up to 100 kg, the absolute increase in live weight increased in comparison with the analogues of group 1 by 1.5 kg, or 2.1% and 0.8 kg, or 1.13 %, feed costs increased by 0.39 units, or 8.99% ($p \geq 0.95$) and 0.13 units, or 3.04%. The results obtained allow us to conclude that the use of the amino acids lysine and methionine in general diets can help to reduce the cost of feed per unit of growth and this helps to save feed, and also helps to increase the efficiency of pork production. As a result of the control slaughter of the experimental animals, we studied the meat qualities of the experimental young animals, which are presented in Table 2. The presented results in Table 2 indicate that the qualitative indicators were at the same level and did not have significant differences ($p \leq 0.95$). It is worth noting that when fattening up to 100 kg in meat of gilts of group 3, the meat yield was higher in comparison with groups 1 and 2 by 0.5 and 0.8%, and the protein-quality indicator (BPC) i.e. the ratio of the essential amino acid tryptophan to the essential amino acid oxyproline is 0.02 and 0.08% higher in animal meat. According to the area of the muscle eye, the meat of animals of the 1st group is the best indicator, which is higher than in the 2nd and 3rd groups by 0.5 and 0.4 cm^2.

In addition, we analyzed the amino acid scores of pork obtained from experimental gilts. For comparison, three samples were taken from half carcasses of groups 1, 2, and 3. For a comparative assessment, the content of the most deficient amino acids of tryptophan, methionine + cystine and lysine was studied in meat.

By comparing the reference protein recommended by the FAO/WHO with experimental data, the following results were obtained: 1.29 $g.100g^{-1}$ of protein was contained in meat obtained from tryptophan group 1 animals; methionine + cystine – 3.58 $g.100g^{-1}$ protein; lysine – 5.31 $g.100g^{-1}$ protein, which is less in comparison with analogues of groups 2 and 3 in tryptophan – by 3.9 and 7.7%; methionine + cystine – by 2.2 and 2.8%; lysine – by 1.9 and 6.0%, respectively.

The studies presented showed that in the meat of animals of group 1, the amino acid rate was the smallest and amounted to 96.9%, and in the meat of animals of groups 2 and 3 – 103.8 and 105.6%. In order to establish the nutritional value of pork obtained during research, an organoleptic assessment of broths was carried out on a 5-point scale with the participation of 15 tasters. Based on the appearance of the meat broths, the tasters determined that the broth prepared from pork of the 1st group scored the highest score – 5.49.

Assessing the appearance of meat broths, experts found that the broth obtained from the meat of gilts of the experimental group I scored the highest score – 5.49. At the same time, it was inferior in terms of aroma, taste, richness and overall rating, the broth of group 3 received the highest scores. The second place was established during the tasting of the broth of group 2 (Table 3).

Tasters found that in terms of appearance, aroma, taste, texture, juiciness and overall rating, the best results among the studied groups were boiled meat of gilts of group I. The second place, according to the general assessment, was obtained by the meat of gilts of the 3rd group. In **Smith (1999)**, it is noted that increasing the content of amino acids, including metinin and cystine, in the diets of pigs affects not only the improvement of digestibility of nutrients, but also the energy value of the diet.

Table 1 Dynamics of live weight of experimental gilts, kg (n = 16).

Group	Live weight					
	when fattening	at the age of months			when removing from fattening kg	
		4	5	6	100	120
1	29.7 ±0.40	45.1 ±0.95*	64.1 ±1.22*	84.2 ±1.55*	99.8 ±3.18	119.9 ±3.28
2	29.8 ±0.46	42.3 ±0.98	60.5 ±1.08	78.8 ±1.68	100.4 ±2.44	119.3 ±3.68
3	29.9 ±0.48	44.1 ±0.98	62.4 ±1.06	83.2 ±1.14*	100.2 ±3.02	120.3 ±4.20

Table 2 Qualitative indicators of pork obtained from experimental gilts.

Group	Muscle eye area, cm	Ham weight, kg	Meat yield, %	BKP, %
When fattening to mass 100 kg				
1	28.3 ±0.20	9.22 ±0.30	53.0 ±1.0	8.00
2	27.8 ±0.22	9.16 ±0.30	52.7 ±1.0	7.92
3	27.9 ±0.24	9.22 ±0.20	53.5 ±1.3	8.02
When fattening to mass 120 kg				
1	30.3 ±0.18	11.42 ±0.40	52.6 ±1.0	7.90
2	29.9 ±0.28	11.39 ±0.40	49.8 ±1.0	7.72
3	30.3 ±0.20	11.32 ±0.45	52.6 ±1.2	7.80

Table 3 Organoleptic evaluation of meat broth, score.

Indicator	Group		
	I experienced	II experienced	III experienced
Appearance	5.49	5.38	5.44
Aroma	4.61	4.64	4.68
Taste	4.53	4.66	4.73
Richness	4.47	4.55	4.66
Overall raiting	4.78	4.81	4.88

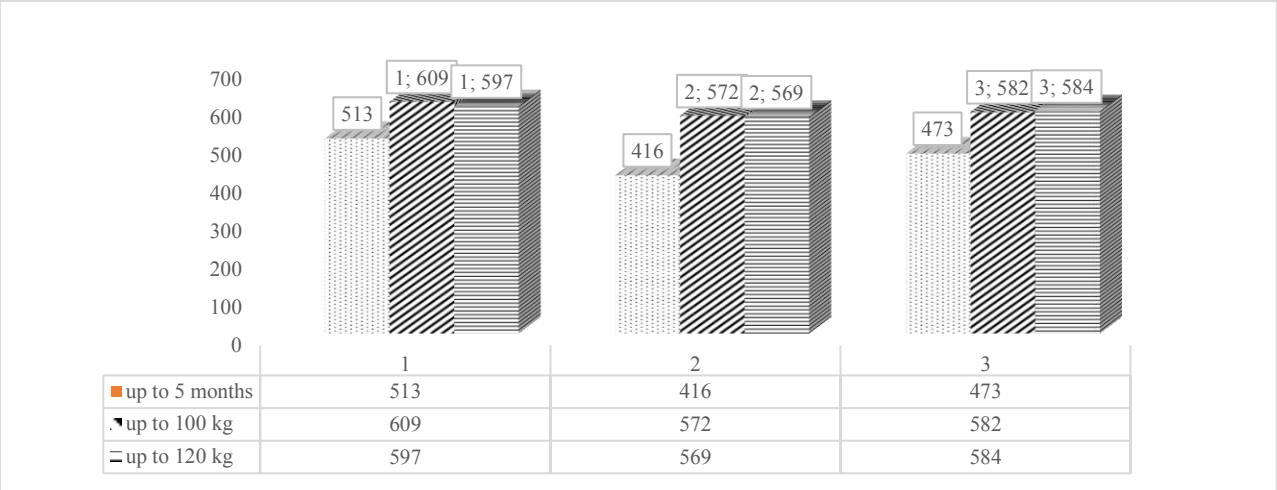

Figure 2 The average daily gain in live weight of experimental guinea pigs (g).

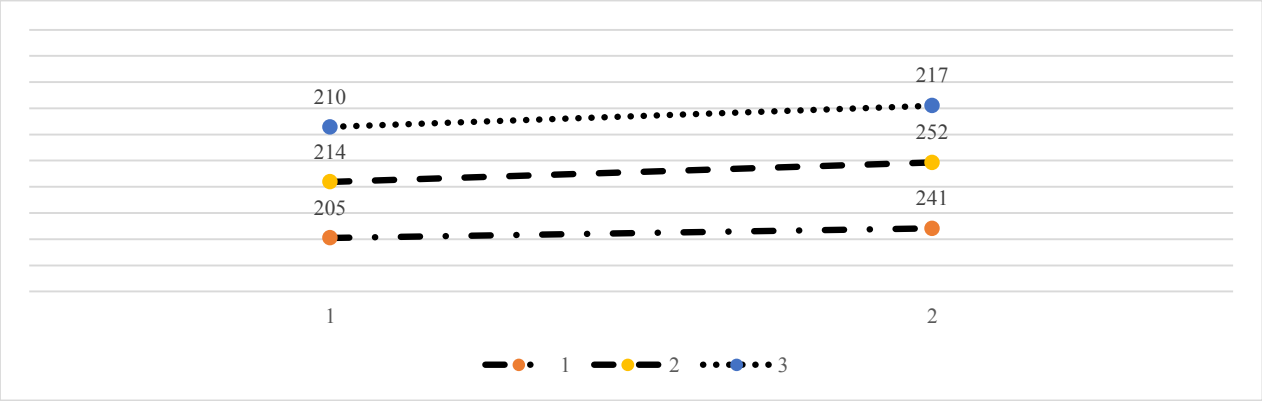

Figure 3 Age of achievement of live weight of 100 and 120 kg, days.

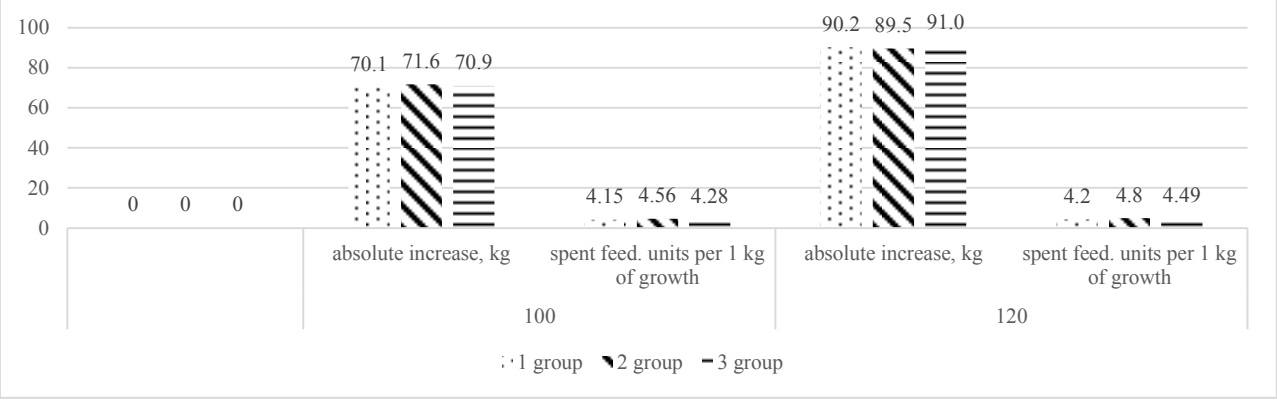

Figure 4 Feed costs and absolute increase in live weight depending on the period from the feed.

The lack of lysine is completely eliminated when introduced into the pre-start feed of piglets 2 – 4 months of age with 20% of its addition. When granulating feed for pigs, it is necessary to take into account the temperature regime, it should not exceed 50 °C since at a higher temperature there is a connection of sugar with amino acids **(Shkatov, 2013)**.

The data obtained by us in the experiment are consistent with earlier works of **(Alikaev et al., 1967; Komlatsky, 2012; Zimina, 2012; Sazonov, 2013; Sanchez, 2012)** in which there is a high efficiency from the use of various components of the diet for its better digestibility, including the use of lysine in combination with methionine.

Today, the world pays great attention to the production of environmentally friendly food, including animal origin. In this regard, studies aimed at studying the increase in pork production due to the introduction of environmentally safe feed additives based on amino acids are very relevant **(Komlatsky, 2012)**.

However, in the work of **Varley (2012)** it is noted that with the modern development of the pig industry, it is impossible to abandon the use of atibiotics in some cases. It is especially important to use feed antibiotics in various feed disorders to control the health of animals and reduce the impact of pathogenic microflora.

A number of Russian and foreign researchers note that for the growing organism of pigs, it is necessary to use an increased level of amino acids in the diets of feeding, including methionine and lysine. This contributes to a significant increase in metabolic processes in their body, which significantly increases the living mass in comparison with analogues that did not receive these amino acids **(Ryadchikov et al., 2000; Ryadchikov et al., 2010; Omarov, 2007, Omarov et al., 2010; Ettle, et al., 2004; Moreira et al., 2004; Stein et al., 2007, Main et al., 2008, Niyazov N.S.-A., et al., 2019)**.

Cheryukanov, 2013 notes that reducing the level of raw protein in the diets of pigs in the growing period from 17.2 to 12.5%, and in the first period of fattening from 15.0 to 11.5% and the second period of fattening from 13.0 to 10.5% is possible if the diets are balanced with synthetic amino acids lysine, threonine and methionine in an amount 24-37% higher in comparison with detailed standards of the all-Russian Institute of animal Husbandry. This contributes not only to the normalization of the level of elemental composition of the blood, but also has a positive effect on the growth of meat productivity. Thus, the live weight of pigs increased by 18.6 and 14.3%, and in the second period of fattening – by 16.5 and 14.33%, than in the control group. This is consistent with the results obtained in our experiments.

Thus, justifying all of the above, we can conclude that the study and justification of the use of synthetic amino acids lysine and methionine in pig feeding will contribute to the development of diets aimed at increasing the productivity of animals. These developments will not only increase the productivity of animals, but can also be used in the development of technologies for obtaining safe food products of animal origin.

CONCLUSION

As a result of the experimental work, it was found that the lack of amino acids such as lysine and methionine in pig diets adversely affects the productivity of animals. It has been experimentally proved that when fattening pigs to 100 and 120 kg of live weight, it is necessary to maintain the amino acid content at the optimal level due to the introduction of synthetic amino acids, which provides an increase in live weight gain and a decrease in the age of removal from fattening. An increase in the content of a balanced amount of amino acids in the diet contributed to an improvement in the balanced composition of their meat. Thus, no significant differences ($p \leq 0.95$) were found in the nutritional value of the resulting meat.

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THE COMPARISON OF HPLC AND SPECTROPHOTOMETRIC METHOD FOR CHOLESTEROL DETERMINATION

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ABSTRACT

The present study was carried out to compare two different analytical methods (HPLC and spectrophotometric) for determination of cholesterol content in milk while cholesterol in food is important not only for the nutritional value setting of foods but also due to the validation of a fast, reliable and economical method for studying the possible mechanism of its reduction. Spectrophotometric determination of cholesterol content was based on the Liebermann-Burchard (LB) reaction among cholesterol, ethyl acetate, acetic anhydride, plus concentrated H_2SO_4 and measuring absorbance of formed color at 625 nm. HPLC method was performed by column chromatography on reverse phase C_{18} with DAD detection at 205 nm. The methods were applied to the milk sample. The achieved LOD and LOQ for HPLC were 2.13 $mg.kg^{-1}$ and 6.45 $mg.kg^{-1}$, respectively, while for spectrophotometric method were 12.55 and 38.04 $mg.kg^{-1}$. The difference between cholesterol content determined by both methods was statistically insignificant at p <0.05. Therefore, it can be concluded that both methods are suitable for determination of cholesterol content in milk, however, HPLC method exhibited higher sensitivity and lower limits of detection or quantification, respectively.

Keywords: cholesterol; HPLC; spectrophotometry; analysis; milk

INTRODUCTION

Cholesterol is a key compound in most biological systems. It is an essential compound in cellular membrane functions of animals and the precursor of important endogenous substances. In humans, cholesterol is obtained from two sources: endogenous synthesis and exogenous ingestion from food **(Ramalho, Casal and Oliveira, 2011)**.

From a nutritional point of view, cholesterol is not found in significant amounts in plant sources, is mostly present in foods of animal origin, namely cheese, egg, beef, pork, poultry, fish, and shrimp. High levels of low-density lipoprotein cholesterol are a major cardiovascular risk factor. Once dietary cholesterol intake is increasing, the plasma cholesterol levels rise and consequently increases the risk of cardiovascular diseases and atherosclerosis **(Albuquerque et al., 2016)**.

Multiple methods have been developed for cholesterol levels determination. According to **Li et al. (2019)** the methods can be divided into three major categories: 1. classical chemical methods based on the Abell-Kendall protocol, 2. fluorometric and colorimetric enzymatic assays, and 3. analytical instrumental approaches. Cholesterol determination procedures in foods usually involve lipid extraction, separation of cholesterol from interfering components or liberation of cholesterol into the free form, and measurement of isolated cholesterol. A mixture of polar and nonpolar solvents has been suggested to give better cholesterol extraction from food materials because cholesterol in these samples is usually bound by many other biological compounds such as lipoproteins, proteins, and phospholipids, and a multiple extraction approach was thought to be more suitable to remove membrane cholesterol **(Dinh et al., 2011)**. Gas and liquid chromatography are the most suitable methods for cholesterol determination, due to their ability to separate and quantify this compound from other similar ones **(Albuquerque et al., 2016)**. The foremost colorimetric test for the identification of cholesterol is probably the Liebermann-Burchard (LB) reaction, which was first described in 1885 **(Xiong, Wilson and Pang, 2007)**. It includes saponification of cholesterol ester with alcoholic potassium hydroxide, extraction of hydrolyzed cholesterol with hexane followed by evaporation of the solvent, and finally color development with acetic anhydride and concentrated sulfuric acid. However, its use is not accepted for routine tests nowadays since highly corrosive reagents are used **(Li et al., 2019)**. High-performance liquid chromatography (HPLC) has the main advantage of being carried out at relatively low temperatures, thus preventing cholesterol oxidation **(Ramalho, Casal and Oliveira, 2011; Albuquerque et al., 2016)**. In spite of some

drawbacks, such as elevated volumes of solvents and limits of detection and quantification, sample preparation is simple and required a small number of steps (saponification and the choice of extraction solvents are needed for adequate separation and quantification of analytes by HPLC) **(Bauer et al., 2014)**.

Scientific hypothesis

Both HPLC and spectrophotometric method could be acceptable for the determination of cholesterol content in milk.

MATERIAL AND METHODOLOGY

All reagents and standards were of analytical grade. Cholesterol standard was from Sigma-Aldrich with a purity ≥99%. Potassium hydroxide (KOH), concentrated sulfuric acid (H_2SO_4), and acetic anhydride were purchased from Mikrochem (Pezinok, Slovakia). Ethyl acetate, n-hexane, and sodium sulphate anhydrous were purchased from Centralchem s.r.o. (Bratislava, Slovakia). Methanol, HPLC grade was purchased from Fisher Chemical (Loughborough, UK). The cow´s milk (3.5% fat, Tatranská mliekareň a.s., Kežmarok, Slovakia) was bought in a local market.

Sample preparation

HPLC analysis

The samples were prepared according to the modified method of **Borkovcová et al. (2009)**. To the 5.0 g of the sample methanolic solution of KOH (1 mol.L^{-1}) was added and refluxed for 30 min. After cooling, 10 mL of n-hexane and 5 mL of deionized water were added and intensively shaken in a separating funnel. The organic layer was separated into the beaker with 2.0 g of sodium sulphate. The water layer was further washing 2 more times. The hexane solution was evaporated, and the residue was dissolved in 3 mL of ethyl acetate. The solution was filtered using syringe filters with PVDF membrane and particle size 0.45 μm (Agilent Captiva, USA). The prepared solution was directly analyzed by HPLC chromatograph. The calibration curve was performed using seven standard concentrations. A stock solution of cholesterol (1 mg.mL^{-1}) was diluted in methanol to prepare calibration standards at 25, 40, 50, 75, 100, 300, and 350 μg.mL^{-1}.

Spectrophotometric determination

The samples for spectrophotometric determination of cholesterol were prepared similarly to HPLC analysis. The LB color reagent was prepared according to the modified method of **Xiong et al. (2007)**. Ethyl acetate (75 mL), acetic anhydride (60 mL), and concentrated H_2SO_4 (12 mL) were pipetted to the volumetric flask at 0 °C, stirring for 10 min, and storage in the fridge for 3 hours. To the prepared LB reagent 1 mL sample solution was added. After 5 min the absorbance value was recorded at 625 nm for 20 min. The concentration of cholesterol in the sample was calculated from the calibration curve, which was performed using calibration standards. The calibration standards were prepared by dilution of cholesterol in ethyl acetate at 0.1 to 1 mg.

Instrument and chromatographic conditions

HPLC

Chromatography analysis was performed using an Agilent Technologies 1260 infinity system (USA) equipped with a vacuum degasser, a quarterly pump, an autosampler, and the UV-DAD detector. Cholesterol was detected at UV wavelength of 205 nm. Isocratic elution was performed at a flow rate of 1.2 mL.min^{-1} using the mobile phase consisted of water/methanol 5:95 (v/v). The injection volume was 10 μL and the temperature was set at 35 °C. As a stationary phase, a Poroshell 120 EC-C18 column (4.6 x 50 mm, 2.7 μm particle size) was used with the guard column Poroshell 120 EC-C18 (4.6 x 5 mm, 2.7 μm particle size). The results were recorded using the OpenLab CDS software, ChemStation Edition for LC and LC/MS systems (product version A.01.08.108).

Spectrophotometric determination

Spectrophotometric determination was performed using a spectrophotometer Cary 300 UV-Vis (Agilent Technologies, USA). The detection wavelength was 625 nm. The results were determined with Cary WinUV software (software version 4.20(468).

Statistical analysis

Results are expressed as mean ±standard deviation or as percentage. Statistical analysis was performed using Microsoft Exel version 2010. The data were subjected to the Student´s test and the values were considered significantly different when $p < 0.05$.

To obtain validation parameters, the linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and selectivity were determined. The linearity was evaluated according to the correlation coefficient by Pearson (R^2) for linear regression. The LOD and LOQ were calculated considering the signal-to-noise ratio accepted for each limit and the parameters estimated for the analytical curve, according to equations 1 and 2:

$$LOD = 3.3 \times \frac{s}{S} \quad (1)$$

$$LOQ = 10 \times \frac{s}{S} \quad (2)$$

Where s is the estimate of the standard deviation of the equation's linear coefficient, and S is the angular coefficient of the analytical curve **(Bauer et al., 2014)**.

The precision was assessed by the Horrat test, which is the ratio of the method standard deviation and the Horwitz relative standard deviation (equation 3):

$$RSD_{Horwitz} = 2^{(1-0.5logC)} \quad (3)$$

Where C is the analyte concentration in mass percentage **(Ribeiro and Brandäo, 2017)**.

The accuracy was evaluated by recovery studies at one standard concentration level of cholesterol (1 mg.mL^{-1}). Recoveries were evaluated by adding to milk sample aliquots standard solutions of the analytes. After the quantification of the analytes in the fortified samples and in the control, the recovery percentage (% REC) was calculated according to equation 4 **(Bauer et al., 2014)**:

$$\%REC = \left(\frac{Obtained\ conc. - Control\ conc.}{Expected\ conc.}\right) \times 100 \quad (4)$$

Selectivity was evaluated by using the spectra provided by the DAD detector by comparison of the peaks present in the chromatograms of the products with those peaks in the chromatograms of the standards, as described by **Bauer et al. (2014)**.

In order to evaluate the conformity of the results obtained by HPLC and spectrophotometric determination, Moore´s test was used according to **Eckschlager, Horsák and Kodejš (1980)**. The test is applicable if $n_A \neq n_B$ and the range of R_A and R_B is used as a measure of variance. Conformity is tested according to Moore´s criterion (U). Moore´s criterion is calculated according to the equation 5:

$$U = \frac{|\overline{x_A} - \bar{x}_B|}{R_A + R_B} \quad (5)$$

Where x_A is an average value obtained from the first method, x_B is the average value obtained from the second method, and R_A, R_B are the values of variance. The calculated U is compared with the critical value U_α. If $U \geq U_\alpha$, the difference is statistically significant at $p < 0.05$. If $U < U_\alpha$, the difference is not significant and we accept the null hypothesis about the consistency of the results **(Eckschlager, Horsák and Kodejš, 1980)**.

RESULTS AND DISCUSSION

Optimization of the spectrophotometric determination and chromatographic conditions

In color-based methods, the application of the LB reaction is usually the key step after the extraction procedure **(Dinh et al., 2011)**. Cholesterol in the presence of concentrated sulphuric acid and acetic anhydride is oxidized to a conjugated pentaene known as cholestapolyene carbonium ion and this undergoes further reaction to form cholestahexaene sulphonic acid, with a wavelength of absorption of 410 nm **(Adu et al., 2019)**. The LB reaction depends, however, on various factors, such as temperature, time, proportions of reactant, wavelength or exposure of light as described by **Kenny (1952)** or **Essaka (2007)**. Firstly, our study thus investigated the kinetics of LB reaction. We monitored the dependence between the time of reaction and the absorbance of the solution. The results are shown in Figure 1. The absorbance maximum at 625 nm is stable for 20 to 30 min and there is little difference in the measured absorbances. With the increasing time, the absorbance maximum is moving to higher wavelength values (665 to 670 nm), where is also stable. However, a longer time interval is less suitable regarding total analysis time. From the Student´s test, it was observed that the difference between the absorbance values at 625 nm in 20 and 30 min was not significant at $p < 0.05$. The spectrophotometric measurement was thus optimized regarding these results. **Atinafu and Bedemo (2011)** used quite a similar wavelength (640 nm) for the determination of cholesterol in some commercial edible oils. According to **Burke et al. (1974)**, a 30 min reaction time is optimum for spectrophotometric measurement. According to **Kim and Goldberg (1969)**, maximum color development occurs after 15 – 18 min incubation at 30 °C. The other important factor, which has to be considered, is the stability of LB color reagent. **Kim and Goldberg (1969)** stated that the LB reagent is not unstable and it need not be used within a few hours. According to these authors, the reagent is stable for 6 months when stored at 4 °C. On the other hand, some authors using the LB reagent, which was prepared freshly **(Sperry and Brand, 1943; Xiong et al., 2007; Adu et al., 2019)**. Firstly, the stability of LB reagent was measured after 7 hours. After this time the new calibration standards curve was recorded. Based on the Student´s test the differences were statistically insignificant at $p < 0.05$. Statistically insignificant differences were also noticed after 24 and 48 hours. From the results, it was thus obvious that LB color reagent is stable. The reaction is also influenced by the stability of cholesterol solution. The difference between the results obtained with the freshly prepared cholesterol solution and after 21 days was statistically significant at $p < 0.05$ thus the solution was not stable, and the use of freshly prepared solution is recommended.

Because of the slight polarity caused by the hydroxyl group, either normal-phase (NP) or reversed-phase (RP) HPLC can be used for the analysis of cholesterol **(Dinh et al., 2011)**. In our study, we worked with non-polar C_{18} stationary phase and polar mobile phase.

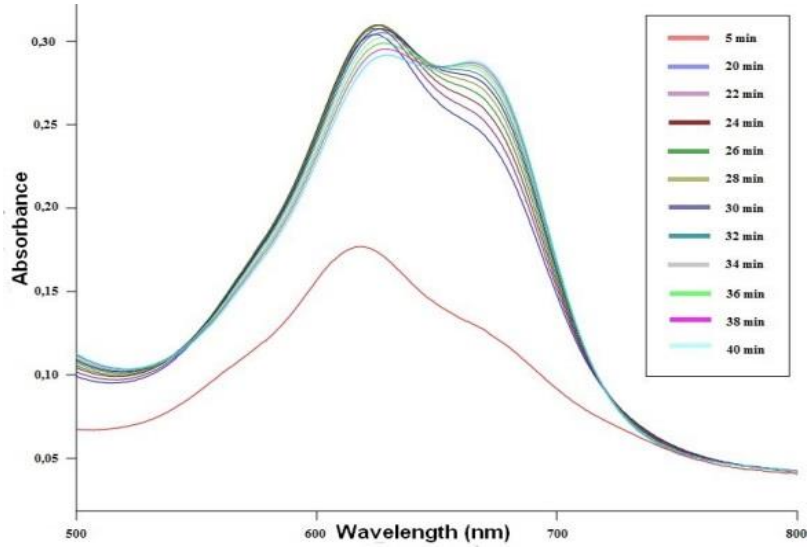

Figure 1 Absorbance spectrum of Liebermann-Burchard reaction.

In literature, there is described a lot of different types of mobile phase composition. For example, **Borkovcová et al. (2009)** used water with methanol 5:95, **Oh, Shin and Chang (2001)** acetonitrile: methanol: isopropanol 7:3:1 or **Bauer et al. (2014)** acetonitrile with isopropanol 95:5. In this work, several mobile phases were tested but the best results were obtained using deionized water with methanol (5:95, v/v). The same conditions were described by **Borkovcová et al. (2009)**. After the optimization procedure, the retention time of cholesterol peak was 5.2 min. The absorbance spectrum (Figure 2) showed that the maximum is obtained at 201 nm, but to avoid the interferences caused by impurities, we used the absorption at 205 nm, because the differences were not statistically significant $p < 0.05$.

Sample analysis and validation

The sample saponification and extraction of cholesterol to non-polar solvent were crucial steps for the analysis of this compound in milk by both methods. The saponification of the lipids has the primordial objectives of removing acylglycerols from the extract of the lipids and hydrolyzing the esters of cholesterol. The reaction can be done after the extraction of the lipids, or by direct saponification **(Bauer et al. 2014)**. These authors also suggested that direct saponification is preferably due to a significantly lower quantity of solvents and shorter preparation time. In our work, we thus used direct saponification followed by the extraction. According to **Ahn et al. (2012)**, three important factors must be considered when selecting a cholesterol extraction solvent: a high solubility of cholesterol, a low efficiency for fat extraction, and hydrophilicity. The most widely used solvents are n-hexane or toluene. Especially hexane has some advantages, such as it is less toxic than other solvents and does not form emulsions as toluene does **(Fletouris et al., 1998)**. The extraction with hexane was performed three times due to increased efficiency, as described **Oh, Shin and Chang (2001)**. Based on these authors, the chromatogram of method, which used hexane as the extraction solvent, had an excellent baseline and no interference was detected. The efficiency of extraction with hexane is also influenced by the presence of water **(Fletouris et al., 1998)**. Therefore, a small amount of water was added to the extraction solvent. The water was then removed by the filtration through anhydrous sodium sulphate. Almost the same steps were also described by **Borkovcová et al. (2009)**.

Based on these modified methods the cholesterol content in milk was analyzed by both techniques. By HPLC the mean content of cholesterol in milk was determined on 92.78 ±9.57 $mg.kg^{-1}$ and by spectrophotometric determination on 84.57 ±10.95 $mg.kg^{-1}$. The 3D record of cholesterol peak in the milk sample is showed in Figure 3.

Ramalho, Casal and Oliveira (2011) determined the mean content of cholesterol in commercial milk samples on 11.6 ±0.2 $mg.100\ mL^{-1}$ by HPLC. According to **Faye et al. (2015)**, the mean values of cholesterol in cow milk are 8.51 ±9.07 $mg.100\ g^{-1}$, which is close to our results.

From the results of **Manzi, Di Costanzo and Mattera (2013)**, the average cholesterol content in Italian cow´s milk is 12.8 ±0.4 $mg.100\ g^{-1}$. Thus, on average, the cholesterol content of whole milk is 12 $mg.100\ g^{-1}$. The variations of values can be attributed to variations in the processing of the milk as well as to differences in the animal breeds, individual characteristics, and intervals between milking, lactation phase, the composition of the animal´s diet, etc. **(Bauer et al., 2014)**.

To obtain the validation parameters, the linearity of both methods was performed by the calibration curves. The linearity is the ability of a method to demonstrate that its results are directly proportional to the concentration of the analyte in the sample, within the linear working range **(Ribeiro and Brandäo, 2017)**. In spectrophotometric determination, the linear range was obtained in the range of cholesterol content 0.1 to 1 mg with the correlation coefficient of 0.9992. In HPLC the linear range was achieved at the cholesterol concentrations at 25 to 350 $mg.L^{-1}$ with the correlation coefficient at 0.9999. This result agrees with **Albuquerque et al. (2016)**, where the linearity was obtained over the range of 0.07-0.4 $mg.mL^{-1}$. The obtained LOD and LOQ for HPLC were 2.13 $mg.kg^{-1}$ and 6.45 $mg.kg^{-1}$, respectively, while for the spectrophotometric method were 12.55 and 38.04 $mg.kg^{-1}$. Thus, from the results, it can be stated that HPLC has better sensitivity than spectrophotometric determination. The almost similar values of LOD and LOQ are reported by **Ahn et al. (2012)** with LOD 2.27 $mg.kg^{-1}$ and LOQ 7.56 $mg.kg^{-1}$. The other important validation parameters are accuracy and precision.

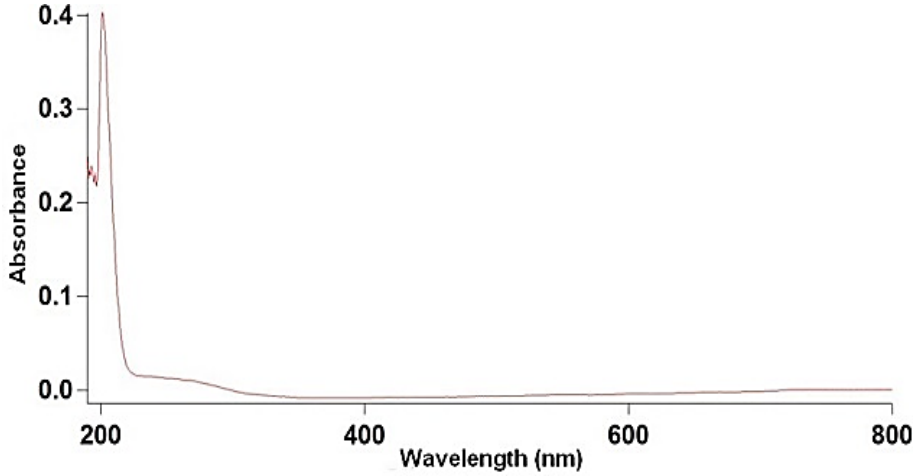

Figure 2 Absorbance spectrum of cholesterol in methanol obtained by UV-VIS spectrophotometer.

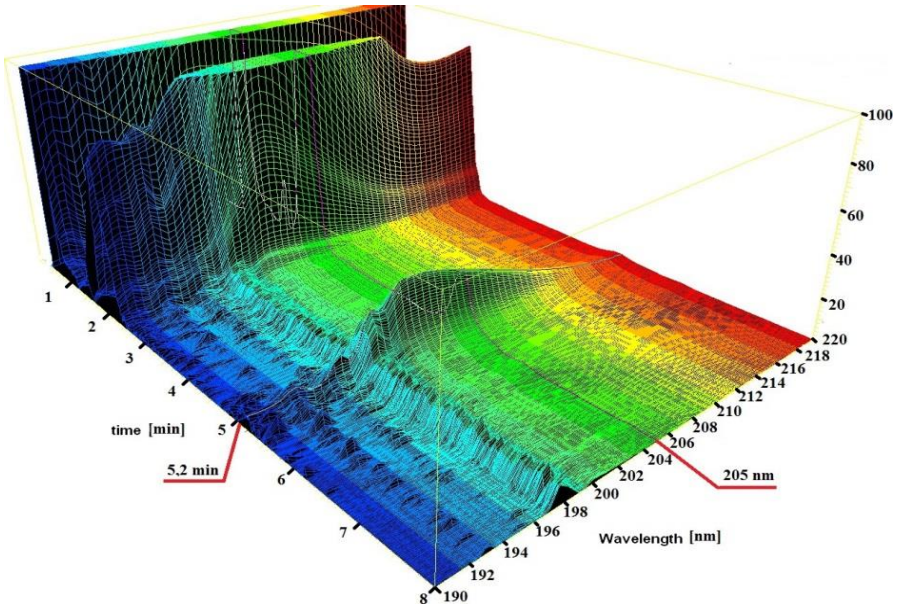

Figure 3 The 3D record of cholesterol peak in milk sample.

Table 1 Comparison of the results obtained from the analysis of cholesterol content in milk by HPLC (Method A) and spectrophotometric determination (Method B).

	Method A[a]	**Method B[b]**
Cholesterol content (mg.kg^{-1} ±SD)	92.78 ±9.57	84.57 ±10.95
LOD (mg.kg^{-1})	2.13	12.55
LOQ (mg.kg^{-1})	6.45	38.04
Recoveries (%)	91.05	85.34
Horrat	1.3	1.45
Slope of calibration curve (b)	433	0.558
Correlation coefficient (R^2)	0.9999	0.9992

Note: [a]n = 6, [b]n = 3, LOD – limit of detection, LOQ – limit of quantification.

The precision refers to the degree of agreement among repeated measurements. Precision is approved when Horrat parameter is less or equal 2 **(Ribeiro and Brandäo, 2017)**. Both methods showed good precision with Horrat value less than 2. Accuracy was obtained by the standard addition method at one concentration level of cholesterol. The recoveries were 85.34% and 91.05% for spectrophotometric determination and HPLC, respectively. Analyte recoveries close to 100% are ideal, but smaller values are admitted if the precision is good **(Bauer et al., 2014)**, and in this case, it is proved by the Horrat values. The selectivity of chromatographic method was proven by the adequate separation of cholesterol with good resolution of the peaks and without co-elution of other compounds in the sample.

The comparison of the propose methods

The comparison of the results obtained from the analysis of cholesterol content in milk by HPLC and spectrophotometric determination is shown in Table 1. For the testing of conformity of the results obtained from both methods, Moore´s test was used according to **Eckschlager, Horsák and Kodejš (1980)**. Based on Moore´s test, the difference between cholesterol content in milk by HPLC and spectrophotometric determination is statistically insignificant at p <0.05 and the null hypothesis of consistency of results is accepted. The resulting mean cholesterol contents in milk determined by these two methods are thus relatively identical. The results showed an 8.8% difference. The cholesterol level in milk can be thus determined by either HPLC or spectrophotometric method. The same conclusion is described by **Essaka (2007)**. Based on his research, the agreement of the values obtained by HPLC and LB reaction with a 16% difference showed that the proposed method was indeed reliable.

As seen from validation parametres, HPLC has some advantages over spectrophotometry. Firstly, LOD and LOQ values are lower thus HPLC is more sensitive. Better sensitivity of HPLC can be seen also from the slope of the calibration curve, where the value is much higher than in spectrophotometric determination. The recoveries were lower in spectrophotometric determination, which can be caused by the different approaches in sample preparation. After saponification and extraction, the sample before spectrophotometry must be reacted with LB reagent, which could lower the recovery. Besides that, the color stability, the issue of temperature dependency, and the turbidity of the final color-developed solution have made colorimetric methods subject to significant concern regarding accuracy **(Dinh et al., 2011)**. According to **Osman and Chin (2006)** HPLC was considered as the method of choice for

cholesterol determination with the lowest LOD and LOQ compare to spectrophotometry and gas chromatography. The performance of spectrophotometer was better than gas chromatography in terms of reproducibility.

CONCLUSION

This study was focused on the comparison of HPLC and spectrophotometric determination of cholesterol content in milk. From the results, the following conclusions can be postulated:

1. The spectrophotometric determination is influenced by the stability and absorbance characteristics of LB reagent.
2. The results obtained from HPLC and spectrophotometric determination differed only in 8.8% thus both methods are suitable for analysis of cholesterol in milk products.
3. HPLC analysis has some advantages over spectrophotometry, mainly higher sensitivity and lower LOD and LOQ values, which makes it more favorable in cholesterol determination in milk.

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PARAMETERS OF ANTIOXIDANT ACTIVITY OF *GALEGA OFFICINALIS* L. AND *GALEGA ORIENTALIS* LAM. (*FABACEAE* LINDL.) PLANT RAW MATERIAL

Olena Vergun, Oksana Shymanska, Dzhamal Rakhmetov, Olga Grygorieva, Eva Ivanišová, Jan Brindza

ABSTRACT

The plant raw material of *Galega officinalis* L. (goat's rue) and *Galega orientalis* Lam. (fodder galega) investigated in this study. These species are known as fodder crops with high productivity of green mass and as medicine plants. The current study was aimed to evaluate an accumulation in dry raw of selected plants the total content of phenolic acids (TPA) and flavonoids (TFC) as compounds with antioxidant activity (AA) by spectrophotometric method. AA by DPPH-method and phosphomolybdenum method (reducing power (RP)) was measured. Study of ethanolic extracts of *G. officinalis* showed accumulation of TPA in different organs in range from 3.65 to 15.17 mg.g^{-1} caffeic acid equivalent (CAE) and TFC from 10.08 to 65.75 mg.g^{-1} quercetin equivalent (QE), AA by DPPH-method from 6.02 to 8.45 mg.g^{-1} Trolox equivalent (TE) and RP of extracts by phosphomolybdenum method from 86.56 to 288.15 mg TE.g^{-1}. In extracts of *G. orientalis* was identified TPA from 3.52 to 18.52 mg CAE.g^{-1} and TFC from 6.09 to 46.72 mg QE.g^{-1}, antioxidant activity by DPPH-method from 6.80 to 8.48 mg TE.g^{-1} and antioxidant capacity by phosphomolybdenum method from 52.52 to 188.51 mg TE.g^{-1}. It was established that less concentration of studied compounds found in the stems for both species. It should be noted that the content of phenolic acids in the leaves was decreased and flavonoids in stems increased during vegetation for both species. Content of phenolic acids in the generative organs and flavonoids in the leaves decreased in raw of *G. orientalis* during vegetation. Pearson's correlation analysis demonstrated very strong relations between TFC and AA by DPPH, TPA and RP, TFC and RP for *G. officinalis* extracts. Very strong correlation in the extracts of *G. orientalis* found between TFC and RP, TPA and RP. Obtained results can be used in the further biochemical and pharmacological study.

Keywords: *Galega officinalis*; *Galega orientalis*; antioxidant activity; flavonoids; phenolic acids

INTRODUCTION

Study of the antioxidant activity and compounds that cause it very widespread and actually in modern biological science **(Carocho and Ferreira, 2013; Kumar, Sharma and Vasudeva, 2017)**. Plant raw material of medicinal **(Adámková, Kouřimská and Kadlecová, 2015; Vergun et al., 2019b)**, food **(Frusciante et al., 2007; Mendelová et al., 2016)**, forage **(Sang et al., 2014; Petrović et al., 2016; Vergun et al., 2018)**, fruit **(Ivanišová et al., 2017; Horčinová Sedláčková et al., 2018; Brindza et al., 2019)** and other plant groups and their products are a valuable source of antioxidant compounds of different nature. Leguminous plants (*Fabaceae* Lindl.) are a perspective group of crops, which ecological and economic function is important in agriculture. It is one of the most important plant families in the production of food for humans and livestock, as well as in the production of industrial products. These crops have provided interesting as forage grasses with high productivity and play an important role as N fixators **(Peiretti, 2009; Teleută et al., 2015)**. Plants from the *Fabaceae* family are of interest in relation to biologically active compounds, especially individuals, in different organs **(Danilčenko et al., 2017)**.

Among economically important leguminous plants can be highlight goat's rue (*Galega officinalis* L.) and fodder galega (*Galega orientalis* Lam.). Plants of species of *Galega* L. are valuable perennial and productive crops with the protein-rich chemical composition of plant raw material **(Baležentienė, 2008)**. They widespread in natural flora and are characterized by high productivity of seeds **(Tkacheva, Vinogradova and Pavlova, 2011)**. Results obtained by **Peiretti (2009)** showed that *G. officinalis* has the potential for large-scale ensiling if plants are harvested at the budding stage or during regrowth. These species cultivated as medicinal plants due to the biochemical composition of plant raw material and as garden plants **(Baležentienė and Spruogis, 2011; Kumar et al., 2012)**. As reported **Kiselova et al. (2006)**, plants of *G. officinalis*

use in traditional phytotherapy due to hypoglycemic and diuretic properties. Also, the hypoglycemic and weight-reducing ability of this species was described in some reports **(Lemus et al., 1999; Hasani-Ranjbar et al., 2009; Shojaee et al., 2015)**.

As described in some reports, plants of *G. officinalis* use in traditional phytotherapy due to hypoglycemic, diuretic properties, and weight-reducing ability **(Modak et al., 2007; Hasani-Ranjbar et al., 2009; Shojaee et al., 2015)**. These plants as a source of metformin use to treat diabetes and use in the pharmacology **(Umashanker and Shruti, 2011; Khodadidi, 2016; Abhati-Evari et al., 2017; Luka, Adoga and Istifanus, 2017)**. Leaves of *G. officinalis* are a source of bioactive secondary metabolites **(Pehlivan Karakas, Sahin and Türker, 2016a)**.

Biochemical composition of *Galega* species raw is ascorbic acid, carotene, soluble sugars, lipids, protein, ash, alkaloids, macroelements, etc. **(Symanowicz and Kalembasa, 2012; Vergun, Shymanska and Rakhmetov, 2012; Shymanska et al., 2017)**. Also, the phytochemicals screening revealed that in aqueous, methanolic, ethanolic and acetone extracts were found flavonoids, tannins, cardiac glycosides, triterpenoids, and steroids. Methanolic extracts of goat's rue significantly improved the lipid profile in a clinical study **(Peirs et al., 2006; Luka, Adoga and Istifanus, 2017)**. As pointed out **Pehlivan Karakas, Yildirim and Türker (2012)**, different extracts of *G. officinalis* showed broad-spectrum activity against both gram-positive and gram-negative bacteria. Moreover, different extracts of goat's rue exhibited cytotoxic, anti-inflammatory and antioxidant activity **(Pehlivan Karakas et al., 2016b)**.

As reported by **Meripõld et al. (2017)**, the first cut of *G. orientalis* advisable to use as a bioenergy crop and the second cut as forage. Also, fodder galega was the object of allelopathic study. Experimental evidence obtained by **Baležentienė (2009)** and **Baležentienė and Kusta (2011)** suggests that shoots of fodder galega are the main source of its allelochemicals, especially at flowering stage. As emphasizes **Ignat, Volf and Popa (2011)**, the main group of biochemical compounds among allelochemicals is phenolic compounds. According to **Symanowicz et al. (2015)**, nitrogen fertilization significantly increased the dry matter yield of fodder galega.

Investigations of oxidative properties of *Galega* species indicated that plant raw material is the source of antioxidants with different natures **(Maslennikov, Chupakhina and Skrypnik, 2014; Shymanska et al., 2018a; Shymanska et al., 2018b)**. Also, high antioxidant activity found in the seed extracts **(Vergun et al., 2019a)**.

Nonetheless, it is necessary to carry out a study with plants of a genus of *Galega* as a source of important biologically active compounds. The aim of this study was to determine the peculiarities of accumulation of compounds with phenolic nature that can detect the antioxidant status of investigated plants as important crops.

Scientific hypothesis

Comparative assessment of the accumulation of phenolic compounds and determination of the antioxidant activity of two species of *Galega* L. genus during vegetation.

MATERIAL AND METHODOLOGY

Conditions of plant growing

The plants were grown in 2017 – 2018 at the experimental fields of the M. M. Gryshko National Botanical Garden of the NAS of Ukraine in the Kyiv city (50°24′55″N, 30°33′45″E).

Biological material

Observation on plants was conducted in the experimental collection of the Cultural Flora Department of M. M. Gryshko National Botanical Garden of the NAS of Ukraine (Figure 1). Plant raw material of two species – *Galega officinalis* and *G. orientalis* were collected in the stages according to Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie (BBCH) coding system **(Meier, 2018)**. According to the BBCH scale, plant samples were taken at the phenological growth stages described for faba bean (*Vicia faba* L.). Four principal growth stages were assigned: leaf development (19 – nine or more leaves infolded), inflorescence emergence (50 – flower buds present, still enclosed by leaves), flowering (65 – full flowering: flowers open on 5 racemes per plant), and ripening (80 – beginning of ripening: seed green, filling pod cavity). For chemical analyses plant raw material was dried at 35 °C for three days **(Müller and Heindl, 2006)**. After this, the samples were milled in the powder condition. All biochemical analyses were done in the Slovak University of Agriculture

Figure 1 *Galega officinalis* L. and *Galega orientalis* Lam. in the stage of flowering.

in Nitra (Slovak Republic).

Sample preparation

For planned analyses, 0.2 g of milling fraction was extracted with 20 mL of 80% ethanol for 24 hours. After centrifugation at 4000 g with Rotofix 32 A (Hettich, Germany) for 20 min, the supernatant was used for measurement (phenolic acids, flavonoids, antioxidant activity by DPPH-method and reducing power of extracts).

Total phenolic acid content (TPAC)

The content of phenolic acids was determined using **Farmakopea Polska (1999)**. 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent, 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of distilled water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/VIS, England). Caffeic acid 1 – 200 mg L^{-1} (R^2 = 0.999) was used as a standard. The results were expressed in mg.g^{-1} caffeic acid equivalents (CAE).

Total content of flavonoids (TFC)

Analise was conducted according to the procedure which was described by **Shafii et al. (2017)**. 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M sodium acetate and 4.3 mL of distilled water. After 30 min. in darkness the absorbance at 415 nm was measured using the spectrophotometer 6405 UV/VIS (Jenway, England). Quercetin 0.01 – 0.5 mg L^{-1} (R^2 = 0.997) was used as the standard and the results were expressed in mg.g^{-1} quercetin equivalents (QE).

Antioxidant activity (AA)

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to **Sánchéz-Moreno, Larrauri and Saura-Calixto (1999)** with slight modification. The ethanol extract (1 mL) was mixed with 4 mL of DPPH solution (0.025 g of radical in 100 mL of ethanol). The absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/VIS, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) 10 – 100 mg L^{-1} (R^2 = 0.983) was used as a standard and the results were expressed in mg.g^{-1} Trolox equivalents (TE).

Reducing power of extracts

Reducing power of extracts was determined by the phosphomolybdenum method of **Prieto, Pineda and Aguilar (1999)** with slight modifications. The mixture of 1 mL of sample, 2.8 mL of monopotassium phosphate (0.1 M), 6 mL of sulfuric acid (1 M), 0.4 mL of ammonium heptamolybdate (0.1 M) and 0.8 mL of distilled water was incubated at 90 °C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/VIS, England). Trolox 10 – 1000 mg L^{-1} (R^2 = 0.998) was used as the standard and the results were expressed in mg.g^{-1} TE.

Statistical analysis

The statistically treated data are given in tables as the arithmetical mean values and their standard errors. Data were submitted ANOVA and differences between means compared through the Tukey-Kramer test (α = 0.05). Correlation analysis was performed using Pearson's criterion.

RESULTS AND DISCUSSION

Antioxidant compounds common nowadays play an important role in protecting factors that explain reducing the risk of different chronic diseases and belong to various classes of biochemical compounds. Phenolic compounds are widespread secondary metabolites in plant extracts, and it possesses various biological activities such as antioxidant, anticarcinogenic, antimicrobial, antiallergic, antimutagenic, anti-inflammatory etc. **(Tatiya et al., 2011; Najafabad Morabbi and Jamei, 2014)**. In addition, they play an important role in plant resistance **(Kulbat, 2016)**. The antioxidant compounds are the natural defense system to protect the plants from abiotic and biotic stresses such as salinity and drought. They play a key role in defense mechanisms against the free radicals which cause the deleterious effect to plant organisms **(Govindaraj et al., 2017)**. A high level of antioxidant agents in medicinal plants can be proposed as an effective therapeutic approach **(Saeed, Khan and Shabbir, 2012)**.

Phytonutrients found in different parts of plants are powerful antioxidants, especially compounds with phenolic nature. Polyphenol compounds from many legumes can be represented by different compounds such as phenolic acids (gallic, ellagic), hydrolyzable tannins **(Akbarirad et al., 2016)**. In some studies, flavonoids indicated as the main components responsible for antioxidant capacity **(Zhang et al., 2012)**. It is a large group of secondary metabolites, which play a variety of significant functions in plants. They play a role as signal molecules, phytoalexins, detoxifying agents, as UV-filters, pollinator attractants etc. **(Samanta, Das and Das, 2011)**. Moreover, as summed up by **Asif (2015)**, flavonoids exhibit a wide spectrum of biological activity such as antioxidant, antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic.

Antioxidant activity of plant extracts can be measured by different methods. Free radical scavenging activity by DPPH method is a simple assay for antioxidant activity evaluation **(Marinova and Batchvarov, 2011; Tatiya et al., 2011; Shekhar and Anju, 2014)**. DPPH radical shows the reduction capability by the decrease in absorbance induced by investigated plant extract. In our study reducing power of plant extracts measured with Trolox as standard while its can be, for example, ascorbic acid **(Tatiya et al., 2011)**.

In Table 1 and Table 2 are shown data of accumulation of total phenolic acids and total flavonoids content in vegetative and generative organs of *G. officinalis* and *G. orientalis* during vegetation. Polyphenol compounds can form several hydrogen bonds and even ionic bonds with most proteins. They modulate the activity of many proteins, involving enzymes, ion channels, etc. As a consequence, many polyphenols are pharmacologically active, being among antioxidants, anti-inflammatory, antibacterial, antifungal, and antiviral **(Wink, 2013)**.

Maslennikov, Chupakhina and Skrypnik (2014) reported that *G. officinalis* leaves contain 3.6 mg GAE.g^{-1} of polyphenol compounds. According to **Tusevski et al. (2014)**, this parameter for *G. officinalis* plants was 32.53 mg GAE.g^{-1}. **Pehlivan Karakas, Sahin and Türker (2016a)** obtained twenty phenolics compounds from methanolic leaves extracts of *G. officinalis*. The total phenolic content, in this case, was 36.69 mg.g^{-1} of dry extract. According to **Sałata and Gruszecki (2010)**, roots and leaves in vegetative stages of plants contained considerably more phenolic acids than the beginning of the flowering period, while in leaf nodes more polyphenolic compounds were marked during flowering than at the vegetative stage. Total phenolic acids in plant raw material of investigated *G. officinalis* plants were in the range from 3.65 to 15.17 mg CAE.g^{-1} depending on the phase of growth (Table 1).

Flavonoids belong to derivates of simple phenols, and their synthesis increases at the stress conditions due to microbial infections, injury, deficiency of nutrients, changing of temperature, etc. **(Kulbat, 2016)**. Flavonoids are biologically active compounds that possess the ability to capture radicals and play a significant role in agriculture and pharmaceutical chemistry as anti-hyperglycemic, anti-cancerous, anti-allergic, anti-viral, immune-stimulating activity **(Sulaiman et al., 2013; Marella, 2017)**.

The level of flavonoid accumulation in raw of *G. officinalis* during vegetation was in the range from 10.08 to 67.75 mg QE.g^{-1}. The study of **Tusevski et al. (2014)** resulted that the concentration of flavonoids in this plant was 8.95 ±0.13 mg CAE.g^{-1}.

As shown in Table 2 phenolic acid content of *G. orientalis* plant raw material ranged from 3.52 to 18.52 mg CAE.g^{-1} during vegetation. The concentration of flavonoids ranged from 6.09 to 46.72 mg QE.g^{-1}. According to **Baležentienė (2009)** report, the highest total content of phenols was determined at the budding stage which was characterized as the most intensive growth period of the plant shoot.

Also, it should be noted that minimal content of phenolic acids and flavonoids in the plant raw material of both investigated species during vegetation was identified in stems. Obtained data showed that higher accumulation of investigated biochemical parameters was different for two species. So, flavonoids content for *G. officinalis* were maximal in inflorescences and phenolic acids – in leaves (inflorescence emergence stage). For *G. orientalis* total content of phenolic acids and flavonoids was maximal in leaves (inflorescence emergence stage).

There are a great number of methods for the determination of antioxidant capacity based on different principles. One of them is the DPPH method that is rapid, simple, and accurate **(Marinova and Batchvarov, 2011; Pisoschi et al., 2016)**. The method is based on the scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the radical solution **(Saeed, Khan and Shabbir, 2012)**. Trolox Equivalent Antioxidant Capacity assay is widely used to evaluate the antioxidant property of investigated products **(Kumar, Sharma and Vasudeva, 2017)**. Figure 2 demonstrates the antioxidant capacity by DPPH-method of plant raw material of *Galega* species during vegetation.

We found that the antioxidant activity of investigated ethanol extracts of *G. officinalis* was in the range from 6.02 to 8.45 mg TE.g^{-1}. For *G. orientalis* extracts this parameter ranged from 6.80 to 8.48 mg TE.g^{-1}.

Also, the antioxidant capacity can be illustrated by the reducing power of investigated extracts as an important indicator. According to some studies, there is some connection between antioxidant activity and reducing power **(Zhang et al., 2012)**. In our experiment extracts analyzed spectrophotometrically through the phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) **(Kumar, Sharma and Vasudeva, 2017; Saeed, Khan and Shabbir, 2012; Ravishankar, Kiranmayi and Prasad, 2018)**. The present study demonstrated that ethanol extracts of *G. officinalis* and *G. orientalis* had antioxidant activity during vegetation ranged between 86.56 – 288.15 and 52.52 – 188.51 mg TE.g^{-1}, respectively (Figure 3).

Correlation analysis was used to explore the relationships between the polyphenols, phenolic, flavonoids compounds and antioxidant capacities (by DPPH and phosphomolybdenum methods) measured for all plant extracts of *Galega officinalis* and *Galega orientalis* (Table 3, Table 4). The results of this study have demonstrated that investigated antioxidant components in two species of *Galega* L. had a correlation between different parameters of an experiment during vegetation. In the period of inflorescence emergence we found a very strong correlation between TPA and TF for both *G. officinalis* and *G. orientalis* (0.985 and 0.950 respectively). Very strong correlation found between AA by DPPH and RP of extracts for *G. officinalis*. The relation between TPA accumulation and RP and TFC and RP was moderate for *G. officinalis* (0.433 and 0.583 respectively). For *G. orientalis* in this period between TPA and RP was strong relation (0.750) and between TFC and RP very strong (0.919). In the period of flowering indicated that very strong correlation found for both species between TFC and RP. Dramatically strong correlation detected between TFC and AA by DPPH (0.999) for *G. officinalis* in this stage. However, in this case for *G. orientalis* found a very weak correlation (0.033). Also, very strong relations found between TPA, TFC and RP for *G. officinalis* (0.924 and 0.851 respectively). In this case values of coefficient of correlation were 0.670 (strong) and 0.953 (very strong) for *G. orientalis*. Different values found for two species regarding the relation between AA by DPPH and RP (very strong for *G. officinalis* and weak for *G. orientalis*).

Table 1 The total content of phenolic acids and flavonoids in plant raw material of *Galega officinalis* L. during vegetation.

Phase of growing	Organ of plant	Total phenolic acids, mg CAE.g^{-1}	Total flavonoid content, mg QE.g^{-1}
Leaf development	aerial part	14.13 ±0.89 ^{a}	44.27 ±2.97 ^{b}
Inflorescence	leaves	15.17 ±0.12 ^{a}	55.61 ±0.75 ^{a}
emergence	stems	6.47 ±0.20 ^{c}	10.08 ±0.94 ^{ef}
	buds	12.44 ±0.07 ^{b}	48.91 ±1.14 ^{b}
Flowering	leaves	11.62 ±0.25 ^{b}	44.91 ±1.12 ^{b}
	stems	3.65 ±0.24 ^{c}	13.18 ±0.86 ^{e}
	inflorescences	7.70 ±0.48 ^{c}	67.75 ±5.05 ^{a}
Ripening	leaves	10.15 ±0.28 ^{b}	32.24 ±0.29 ^{c}
	stems	9.62 ±0.19 ^{b}	24.78 ±0.57 ^{d}
	fruits	5.89 ±0.29 ^{c}	16.86 ±0.55 ^{e}

Note: Means in columns followed by different letters are different at p = 0.05; each value represents the mean of three independent experiments (±SD); GAE – gallic acid equivalents; CAE – caffeic acid equivalents; QE – quercetin equivalents.

Table 2 The total content of phenolic acids and flavonoids in plant raw material of *Galega orientalis* Lam. during vegetation.

Phase of growing	Organ of plant	Total phenolic acids mg CAE.g^{-1}	Total flavonoid content mg QE.g^{-1}
Leaf development	aerial part	16.73 ±0.52 ^{a}	38.79 ±0.83 ^{b}
Inflorescence	leaves	18.52 ±1.64 ^{a}	46.72 ±0.26 ^{a}
emergence	stems	4.37 ±0.19 ^{d}	6.09 ±0.67 ^{e}
	buds	10.89 ±0.77 ^{b}	36.21 ±0.55 ^{b}
Flowering	leaves	16.12 ±0.19 ^{a}	40.09 ±0.48 ^{a}
	stems	3.52 ±0.39 ^{d}	6.74 ±0.51 ^{e}
	inflorescences	7.38 ±0.16 ^{c}	32.63 ±0.79 ^{bc}
Ripening	leaves	12.57 ±0.38 ^{b}	35.59 ±0.48 ^{b}
	stems	4.05 ±0.30 ^{d}	14.47 ±0.46 ^{d}
	fruits	4.25 ±0.24 ^{d}	9.29 ±0.44 ^{d}

Note: Means in columns followed by different letters are different at p = 0.05; each value represents the mean of three independent experiments (±SD); CAE – caffeic acid equivalents; QE – quercetin equivalents.

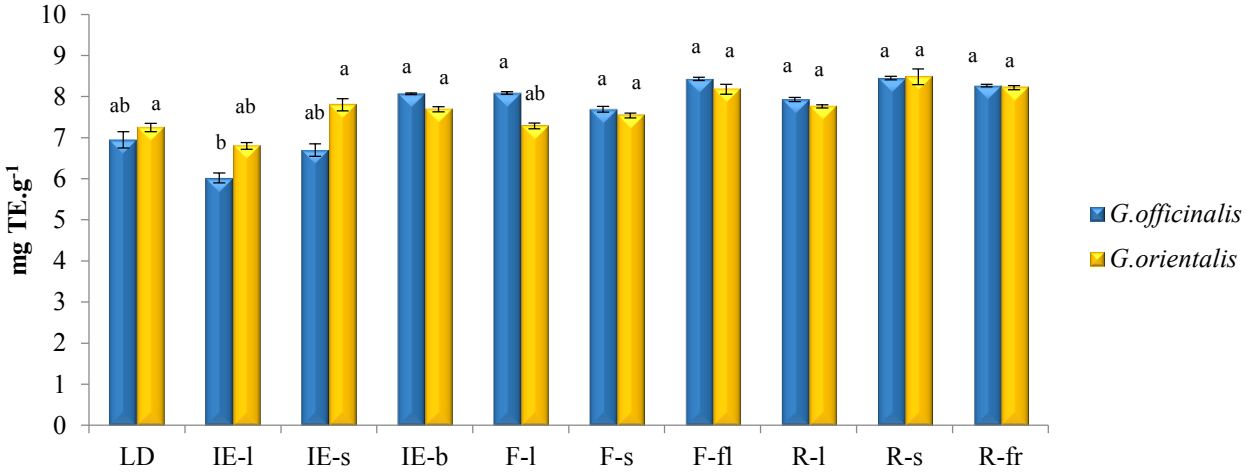

Figure 2 The antioxidant activity of ethanol extracts of *Galega officinalis* L. and *G. orientalis* Lam. by DPPH-method during vegetation. Note: LD – leaf development; IE-l – inflorescence emergence, leaves; IE-s – inflorescence emergence, stems; IE-b – inflorescence emergence, buds; F-l – flowering stage, leaves; F-s – flowering stage, stems; F-fl – flowering stage, inflorescences; R-l – ripening, leaves; R-s – ripening, stems; R-fr – ripening, fruits; means in columns followed by different letters are different at p = 0.05; each value represents the mean of three independent experiments (±SD).

Table 3 Coefficient of correlation between investigated parameters of *Galega officinalis* L. extracts during vegetation.

Characters	TPAC	TFC	DPPH
Inflorescence emergence stage			
TFC	0.985*	1	
DPPH	-0.120	0.055*	1
RP	0.433*	0.583	0.843
Flowering			
TFC	0.587	1	
DPPH	0.548*	0.999	1
RP	0.924*	0.851*	0.825
Ripening			
TFC	0.924	1	
DPPH	-0.247	-0.598*	1
RP	0.551*	0.828*	-0.945

Note: Significant according to the t-test (p <0.05).

Table 4 Coefficient of correlation between investigated parameters of *Galega orientalis* L. extracts during vegetation.

Characters	TPA	TFC	DPPH
Inflorescence emergence stage			
TFC	0.950	1	
DPPH	-0.929*	-0.768	1
RP	0.750*	0.919*	-0.454*
Flowering			
TFC	0.863*	1	
DPPH	-0.476	0.033*	1
RP	0.670*	0.953	0.334
Ripening			
TFC	0.979*	1	
DPPH	-0.941	-0.852*	1
RP	0.880*	0.959	-0.669*

Note: Significant according to the t-test (p <0.05).

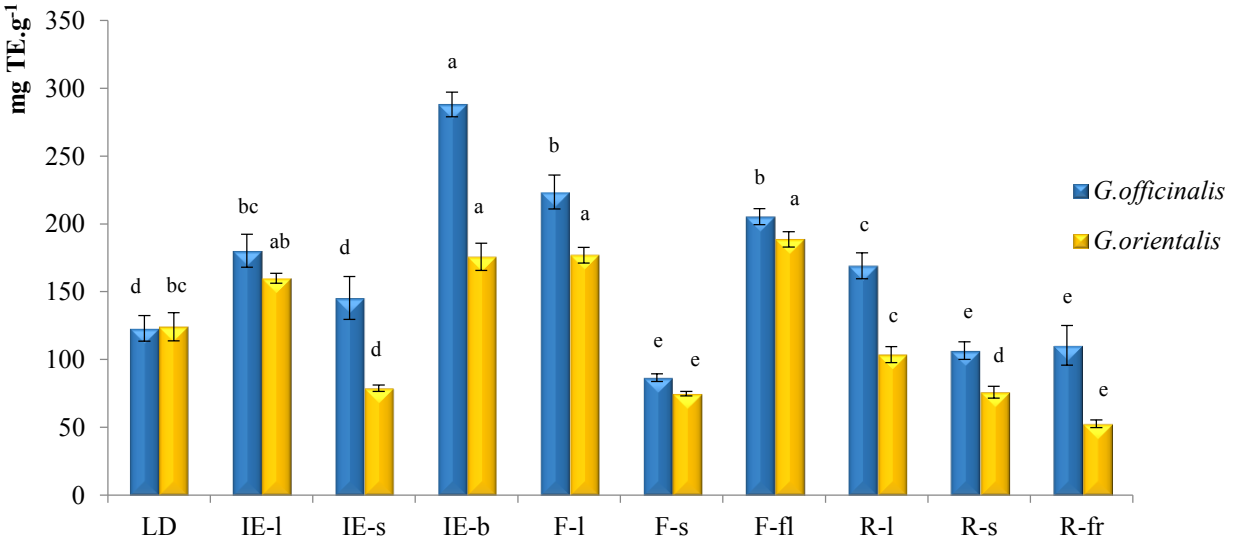

Figure 4 The reducing power of ethanol extracts of *Galega officinalis* L. and *G. orientalis* Lam. during vegetation. Note: LD – leaf development; IE-l – inflorescence emergence, leaves; IE-s – inflorescence emergence, stems; IE-b – inflorescence emergence, buds; F-l – flowering stage, leaves; F-s – flowering stage, stems; F-fl – flowering stage, inflorescences; R-l – ripening, leaves; R-s – ripening, stems; R-fr – ripening, fruits; means in columns followed by different letters are different at p = 0.05; each value represents the mean of three independent. experiments (±SD).

At the period of ripening determined the very strong correlation between TPA and TFC, TFC and RP for both investigated species. Correlation between TPA and RP was moderate for *G. officinalis* (0.551) and very strong for *G. orientalis* (0.880). It should be noted that for both species was found a negative correlation between TPA and AA by DPPH, TFC and AA by DPPH, and between AA by DPPH and RP. The direction of correlation between antioxidant components depends on them nature, thus, different types of phenolics possess different antioxidant activity **(Vamanu et al., 2011)**.

According to previous studies, it should be noted that phenolic extracts exhibited different antioxidant activity that depends on their structure **(Tatiya et al., 2011)**. The study of relationships between phenolic compounds and antioxidant activity demonstrated a significant correlation **(Li, Wu and Huang, 2009)**. Some results, also, not confirmed correlations between the content of phenolic compounds and antioxidant activity **(Vamanu et al., 2011)**. Moreover, according to **Vamanu et al. (2011)**, reducing power and antioxidant activity correlated with extract concentration. In some cases, there wasn't found relationship between phenolic compounds content and antioxidant activity.

CONCLUSION

Based on the results obtained in this study concluded that two investigated species of *Galega* L. as medicine and forage cultures characterized by plant raw material with high antioxidant activity. The maximal content of phenolic acids for both investigated species was found in the leaves in the period at the inflorescence emergence, flavonoids in inflorescences at the flowering stage for *G. officinalis* and in the leaves at the inflorescence emergence period for *G. orientalis*. The least content of phenolic acids and flavonoids identified in the stems of both investigated species. Ethanolic extracts of stems of *G. officinalis* and *G. orientalis* plants exhibited the most antioxidant activity at the ripening stage by DPPH-method. Reducing power of ethanol extracts was higher for *G. officinalis* in the buds, for *G. orientalis* in the inflorescences. Pearson's correlation analysis (at $p < 0.05$) showed very strong values of coefficient of variation between TFC and AA by DPPH (0.999), TPA and RP (0.924), TFC and RP (0.828) for *G. officinalis* depending on the stage of growth. The highest correlation found in extracts of *G. orientalis* between TFC and RP at every investigated stage (0.919, 0.953, and 0.959), between TPA and RP (0.880). Obtained results demonstrated that two investigated species of *Galega* are a good source of antioxidant compounds with polyphenol nature such as phenolic acids and flavonoids. These data can provide further information about an accumulation of flavonoids and phenolic acids in *Galega* spp. raw that possess antioxidant activity and also can be used in pharmacological investigations.

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COMPARISON OF THE PHYSICO-CHEMICAL MEAT QUALITY OF THE BREEDS MANGALITSA AND LARGE WHITE WITH REGARD TO THE SLAUGHTER WEIGHT

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ABSTRACT

The aim of this study was to compare the quality of *musculus longissimus dorsi* in the breeds of Mangalitsa and Large White with regard to the slaughter weight. Large White (LW) breed and White Mangalitsa (Ma) breed were used in the experiment. The system of housing and feeding was the same in both of the monitored breeds. The pigs were fed with the same feeding mixture *ad libitum.* According to the slaughter weight, the pigs were divided into three groups: up to 100 kg, 101 – 110 kg and over 110 kg. The breed Ma had a significantly lower drip loss than the breed LW. Evaluating the color of the meat, the LW breed has showed significantly higher L* (lightness, white ±black) and lower a* (redness, red ± green) values than the Ma breed. Within the chemical meat composition, the Ma breed had a significantly higher water content in MLD compared to the LW breed. Generally, there were no major differences in the meat quality between the Mangalitsa and Large White breeds. Finally it can be concluded that the breed Mangalitsa showed more favorable values of the physico-chemical indicators. Comparing the quality of the meat with regard to the slaughter weight, there were no large differences between individual weight groups. A higher slaughter weight has positively influenced mainly the color of the meat, as pigs weighing more than 110 kg achieved a significantly lower value of L* and a higher value of a* in comparison to pigs of the lower weight. As a positive effect of a higher slaughter weight can be considered its effect on the protein content in the meat, as pigs weighing over 100 kg have a significantly higher protein content in the meat than pigs weighing below 100 kg.

Keywords: Large White; Mangalitsa; pork quality; slaughter weight

INTRODUCTION

Pork quality has become a primary focus for producers, researchers, packers, processors, retailers, and ultimately, consumers **(Newcom et al., 2004)**. In the last few years, consumers and the meat industry have emphasized the descending quality of the pig meat offered, such as a high frequency of pale, soft and exudative (PSE) fault expressed by a high drip loss and low water-holding capacity, unacceptable taste of pork, and a low content of the intramuscular fat **(Florowski et al., 2006)**. The meat quality is evaluated according to the quality parameters, such as the pH, color, or the intramuscular fat content. Meat color is one of the main quality properties, which influences consumer's acceptance, but also reflects the quality of meat **(Alonso et al., 2009)**. Moreover, the meat classification based on the pH and color directly in the cutting plant could help to separate the low-quality meat **(Bednářová et al. 2014)**. There are many factors that influence the final quality of meat, e.g. animal nutrition, transportation, handling and stunning, but it is well-known that the breed itself can affect the pork quality **(Gil et al., 2008; Pascual et al., 2007; Šimek et al., 2004)**.

Meat and the meat products from the autochthonous pigs are highly appreciated by consumers because of their high sensory quality **(Živković et al., 2012)**. A high amount of the intramuscular fat, great concentrations of the heme pigments and high levels of unsaturated fatty acids have been highlighted as some of the most relevant quality aspects in the muscles of the autochthonous pig breeds **(Stanišić et al., 2015)**.

The slaughter weight is considered an important factor determining the economic profitability of the pork production. Some disadvantages caused by the increased slaughter weight are related to the reduced pig performance, feed conversion efficiency, and excessive fat thickness **(Serrano et al., 2008)**. From the point of view of the meat quality, the slaughter weight has shown some effects on the meat color, since the darker and redder colors were found in the pork meat at increasing the slaughter weight **(Ellis et al., 1996; Latorre et al., 2004)**.

Additionally, the intramuscular fat content was found to increase with the increased slaughter weight from 100 to 130 kg **(Weatherup et al., 1998)**. However, the slaughter weight has not shown any effects on the total protein, salt-soluble protein, and instrumental colors or marbling scores **(Serrano et al., 2008; Sutton et al., 1997)**.

Considering the fact that quality of pork may be significantly influenced by a genotype, but also by the weight at slaughter, the aim of this study was to evaluate the physico-chemical parameters of pork of the Mangalitsa and Large White breeds with regard to the slaughter weight.

Scientific hypothesis

We have assumed that the Mangalitsa breed meat will have better physico - chemical parameters compared to the Large White breed and that a higher slaughter weight will have a positive impact on the meat quality indicators.

MATERIAL AND METHODOLOGY

Biological material

In the experiment, a total of 20 pigs were analysed, 12 pigs of the breed Large White (LW) and 8 pigs of the breed White Mangalitsa (Ma). The pigs were bred on the pig farm in the village of Žirany (Slovakia).

Feeding and rearing conditions

The housing and feeding systems were the same for both breeds. The pigs were bred under the intensive breeding conditions, while they were housed in groups so as to comply with the requirements for the minimum housing area according to Government Regulation (SR) no. 735/2002 Coll., laying down the minimum standards for the protection of pigs **(NCSR, 2002)**. There was a concrete floor in the pens, covered with the wheat straw. The temperature in the stables was kept at 18 – 20 °C. The air exchange in the stables was realized by a vacuum ventilation system. Water and feed intake in both breeds was *ad libitum.* Feeding of the pigs was provided by the automatic feeders designed for a dry compound feed, which was granulated in order to reduce dustiness. Nipple drinkers were used for watering. The nutritional composition of the used commercial feed mixture was as follows: crude protein 164.63 $g.kg^{-1}$, crude fat 36.25 $g.kg^{-1}$, crude fiber 48.89 $g.kg^{-1}$, ash 39.69 $g.kg^{-1}$, nitrogen free extract 710.53 $g.kg^{-1}$, metabolisable energy 13.10 $MJ.kg^{-1}$ (the data represent the proportion of individual components in the dry matter of the analyzed feed).

Sampling

After reaching the slaughter weight, the pigs were transported to the Experimental Center of Livestock at the Department of Animal Husbandry of the Slovak University of Agriculture in Nitra, where they were killed and subsequently analyzed. The slaughter was realized according to Government Regulation (SR) no. 432/2012 of the Coll. Of the Slovak Republic, establishing the protection of animals during the slaughter **(NCSR, 2012)**. The meat quality parameters were evaluated in the longest back muscle MLD (*musculus longissimus dorsi*) at the level of the last thoracic vertebra in the right carcass half.

Analysis of the physical indicators

The values of pH 45 minutes (pH_{45}) and 24 hours (pH_{24}) *post mortem* were measured by the pH meter HI99161 (Hanna Instruments, Romania) in the units $-\log_{10}[H^+]$. The electric conductivity was determined 45 minutes (EC_{45}) and 24 hours (EC_{24}) *post mortem* by using the instrument Quality Meter (Tecpro, Germany) in the unit $mS.cm^{-1}$. The drip losses in MLT were measured from 24 to 48 h *post mortem* by the method according to **Honikel (1998)**. The meat color was measured in MLD 24 hours *post mortem* by using the spectrophotometer CM-2600d with the CIE Lab space and illuminate D65 (Konica Minolta, Japan). **Commission Internationale de l'Eclairage (1975)** determined the following color coordinates: L* (lightness, white ± black), a* (redness, red ± green) and b* (yellowness, yellow ± blue). The values were recorded from the average of three random readings across the muscle surface.

Analysis of the chemical indicators

The basic chemical parameters were determined from a homogenized muscle sample *musculus longissimus dorsi* by the FT IR method (FourierTransform InfraRed) using the Nicolet 6700 device (Thermo Scientific, USA). The content of water (%), protein (%), intramuscular fat - IMF (%) and cholesterol ($mg.100g^{-1}$) were determined in the meat sample.

Statistical analysis

Statistical analysis of the obtained results was performed in the **IBM (2011)** SPSS Statistics 20 Program (IBM corp., New York). The Univariate Analysis of Variance (UNIANOVA) was used to assess the effect of genotype and the slaughter weight on the monitored meat quality parameters, with testing the contrasts by means of the Scheffe test at the level of significance $p < 0.05$. The analysis was performed according to the following model equation:

$$Y_{ijk} = \mu + B_i + SW_j + e_{ijk}$$

where Y_{ijk} is an indicator of the meat quality, μ is the overall mean, B_i is the fixed effect (n = 2; Mangalitsa, Large White), SW_j is the fixed effect slaughter weight (n = 3; SW up to 100 kg, SW from 101 to 110 kg, SW over 110 kg), e_{ijk} is the random error. The Pearson correlation coefficient was used to calculate the correlation dependencies between the selected monitored indicators.

RESULTS AND DISCUSSION

Comparison of the physical meat quality indicators in MLD of the slaughtered pigs is shown in the Table 1. Analysis of the physical indicators has shown that the breed and the live weight have any effect on pH_{45}, pH_{24}, EC_{45}, EC_{24}, and the color range b*. Mangalitsa achieved higher pH_{45} values (6.26 ±0.20) compared to Large White (6.12 ±0.22). Similarly, **Lípová et al. (2019)** and **Tomović et al. (2016)** found out that Mangalitsa achieved higher pH_{45} values than its crossbreeds and the Large White breed. The highest pH_{45} values were reached by the pigs with a live weight over 110 kg (6.32 ±0.32).

Table 1 Comparison of the physical meat quality indicators *m. longissimus dorsi.*

Parameter	Breed (B)		Slaughter weight (SW)			Significance		
	Ma (n = 12)	LW (n = 8)	to 100 kg (n = 4)	101 – 110 kg (n = 12)	over 110 kg (n = 4)	B	SW	R^2
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	*Mean ± SD*	*Mean ± SD*			
pH_{45}	6.26 ±0.20	6.12 ±0.22	6.21 ±0.10	6.17 ±0.21	6.32 ±0.32	n.s.	n.s.	0.15
pH_{24}	5.63 ±0.07	5.69 ±0.12	5.61 ±0.03	5.68 ±0.11	5.63 ±0.04	n.s.	n.s.	0.13
EC_{45}	3.50 ±0.34	3.38 ±0.32	3.45 ±0.40	3.42 ±0.36	3.55 ±0.17	n.s.	n.s.	0.05
EC_{24}	11.32 ±3.10	11.08 ±5.07	12.00 ±3.23	11.98 ±4.26	8.15 ±1.56	n.s.	n.s.	0.23
Drip loss	7.57 ±0.88	10.13 ±2.72	7.89 ±0.14	9.15 ±2.73	7.64 ±0.19	*	n.s.	0.35
CIE L*	51.71 ±2.84	58.75 ±1.85	54.85 ±2.16^b	56.03 ±4.40^b	49.68 ±0.82^a	***	**	0.85
CIE a*	3.52 ±1.96	0.59 ±0.65	1.32 ±0.29^a	1.68 ±1.90^a	5.38 ±0.08^b	***	***	0.87
CIE b*	10.79 ±1.22	10.44 ±0.42	10.43 ±0.79	10.48 ±1.11	11.38 ±0.28	n.s.	n.s.	0.15

Note: Ma: Mangalitsa, LW: Large White, n.s.: not significant, *: p <0.05, **: p <0.01, ***: p <0.001, different letters in the same row indicate significant differences among the mean values (p <0.05), R^2: coefficient of determination.

24 hours after slaughter, the pH_{24} values dropped to 5.63 ±0.07 in Mangalitsa and to 5.69 ±0.12 in Large White. Similar pH_{24} values were found in MLD in the breed Ma (5.69 ±0.07) and in the hybrids of Ma x LW (5.68 ±0.11) by **Lípová et al. (2019)**. **Alonso et al. (2009)** found the pH_{24} values of various commercial hybrids at the level of 5.70 - 5.74. Contrary to these findings, **Stanišić et al. (2015)** found higher MLD muscle acidification 24 hours *post mortem* in Mangalitsa at the level of 5.47 ±0.07 and in Landrace at the level of 5.47 ±0.10. In accordance with the results of our study, **Ba et al. (2019)** did not detect any significant differences in pH_{24} values in relation to the pig weight.

Electric conductivity, 45 minutes after slaughter (EC_{45}), was recorded in the breed Ma (3.50 ±0.34 $mS.cm^{-1}$) and LW (3.38 ±0.32 $mS.cm^{-1}$). After 24 hours, there was a significant increase in the electric conductivity (EC_{24}) to 11.32 ±3.10 $mS.cm^{-1}$ for Ma and 11.08 ±5.07 $mS.cm^{-1}$ for LW. Lower EC_{24} values in the breed Ma (9.31 ±1.91 $mS.cm^{-1}$) and the crossbreeds Ma x LW (10.86 ±2.25 $mS.cm^{-1}$) were found by **Lípová et al. (2019)**. In the commercial hybrids, **Mörlein et al. (2007)** found in the study the value EC_{24} at the level 6.24 ±2.32 $mS.cm^{-1}$. In assessing the meat quality deviations, the EC_{24} values at the level of 7 $mS.cm^{-1}$ and 9 $mS.cm^{-1}$, respectively, were used as the criterion for the PSE meat (pale, soft, exudative). It follows from the above that some individuals of both monitored breeds have shown deteriorated meat quality in this indicator. In terms of comparing pigs by their live weight, the EC_{24} values were decreasing with the weight gain.

In the drip loss indicator, the Ma breed (7.57 ±0.88%) had a significantly lower drip loss (p <0.05) than the LW breed (10.13 ±2.72%). No significant differences were found within the weight groups and the confidence of estimation was 35% (R^2 = 0.35). **Lípová et al. (2019)** found similar drip loss values for the Ma breed (7.15 ±2.99%) and the higher ones for the Ma x LW hybrid (8.22 ±2.78%). **Mörlein et al. (2007)** and **Fischer et al. (2000)** found out that the smallest drip loss was reported by the crossbreeds that contained the breed Duroc.

Color is considered an important indicator of the pork quality, as it is one of the most important characteristics affecting consumers´ ratings of meat **(Valous et al., 2010)**. The analysis of the L* values has shown that both the breed (p <0.001) and the live weight (p <0.01) had a significant effect on the meat lightness, with a confidence estimate of 85 (R^2 = 0.85). The LW breed had a lot lighter meat than the Ma breed (58.75 ±1.85 vs. 51.71 ±2.84) and the pigs weighing over 110 kg had significantly darker meat than the lower weight pigs (p <0.05). The a* indicator, representing the meat redness, has also shown a significant effect of the breed (p <0.001) and the live weight (p <0.001), the reliability of the estimate was at 87% (R^2 = 0.87). Meat of the Ma breed was redder compared to the LW breed (3.52 ±1.96 vs. 0.59 ±0.65) and the pigs weighing over 110 kg had significantly redder meat compared to the lower weight pig groups (p <0.05). Neither the breed nor live weight had a significant effect on the indicator b*, which represents the yellowness of the meat. Several authors have found out, when comparing the meat color of the Ma breed with other breeds, respectively with the hybrids of the mangalitsa breed, that the meat of the Ma breed is darker and redder, which is in accordance with our results **(Lípová et al., 2019; Tomović et al., 2016; Tomović et al., 2014)**. **Ba et al. (2019)** comparing the meat color depending on the slaughter weight of the pigs found any significant differences in the meat lightness (L*). In the a* and b* indicators pigs with a higher slaughter weight (120 kg) had significantly redder and yellower meat than the pigs weighing 100 kg. Similarly, **Latorre et al. (2004)** found a higher a* value in MLD of the pigs killed at a higher weight (132 kg) compared to the pigs that were killed at a lower weight (116 kg). Contrary to our results, **Correa et al. (2006)** and **Serrano et al. (2008)** found no significant differences in the meat color depending on the weight of the slaughter pigs.

The basic chemical composition and cholesterol content are shown in the Table 2. The Ma breed had a significantly higher water content in MLD (p <0.05) compared to the LW breed (70.42 ±0.41% vs. 69.22 ±1.51%). However, the slaughter weight have any effect on this indicator and the reliability of the estimate was at the level of 31% (R^2 = 0.31). In contrast, the genotype (n.s.) have any influence on the the protein content of MLD, but the slaughter weight (p <0.05), with a confidence estimate of 53% (R^2 = 0.53). Pigs weighing up to 100 kg had a significantly lower protein content in meat (24.32 ±0.01%) than the pigs of higher weight. The weight categories 101 – 110 kg and over 110 kg had similar

Table 2 Basic chemical composition of the meat and cholesterol content in *m. longissimus dorsi*.

Parameter	Breed (B)		Slaughter weight (SW)			Significance		
	Ma (n = 12)	LW (n = 8)	to 100 kg (n = 4)	101 – 110 kg (n = 12)	over 110 kg (n = 4)	B	SW	R^2
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	*Mean ± SD*	*Mean ± SD*			
Water	70.42 ±0.41	69.22 ±1.51	70.23 ±0.59	69.72 ±1.42	70.29 ±0.18	*	n.s.	0.31
Protein	24.64 ±0.35	25.02 ±0.27	24.32 ±0.01[a]	24.92 ±0.31[b]	24.87 ±0.37[b]	n.s.	*	0.53
IMF	1.34 ±0.38	0.98 ±0.24	1.56 ±0.20	1.07 ±0.40	1.22 ±0.03	n.s.	n.s.	0.35
Cholesterol	44.17 ±5.77	40.00 ±2.14	47.50 ±0.58	40.50 ±5.45	43.50 ±0.58	n.s.	n.s.	0.33

Note: Ma: Mangalitsa, LW: Large White, IMT: intramuscular fat, n.s.: not significant, *: p <0.05, different letters in the same row indicate significant differences among the mean values (p <0.05), R^2: coefficient of determination.

protein content in MLD (24.92 ±0.31% and 24.87 ±0.37%).

The opposite tendency of the slaughter weight influence on the protein content was found by **Ba et al. (2019)** and **Raj et al. (2010)**, who recorded a significantly lower protein content in the pigs of the greater weight (110 – 130 kg) than in the pigs of the lower weight (90 – 110 kg). The differences between breeds were not conclusive (Ma: 24.64 ±0.35%, LW: 25.02 ±0.27%). **Parunović et al. (2013)** found a higher proportion of water in MLD of the Swedish Landrace (72.70%, p <0.001) than in the Swallow-bellied Mangalitsa breeds 64.30% and the White mangalitsa 62.70%, while the difference in water content among the mangalitsa breeds was not significant. **Kim et al. (2016)** found the water content in MLD for the Berkshire breed at the level of 75%. **Holló et al. (2003)** found that different slaughter weight had no effect on the water content of the MLD muscle of the Mangalitsa breed. The water content was 68.80 ±2.01% in the pigs weighing 91.64 ±2.41 kg and 68.96 ±2.60%, in those with the weight of 114.14 ±7.70 kg, which is comparable to our results.

Regarding the protein content in MLD, the differences between the breeds were not significant (Ma: 24.64 ±0.35%, LW: 25.02 ±0.27%). Contrary to our results, several authors found a lower protein content in the Mangalitsa breed, ranging from 19.50% to 21.83% **(Tomović et al., 2016; Stanišić et al., 2015; Parunović et al. 2013; Holló et al., 2003)**. Vice versa, **Lípová et al. (2019)** found a similar protein content in Mangalitsa (24.15 ±0.66%). Also, in the meat breeds and crossbreeds, there are not clear results of protein content in MLD. **Tomović et al. (2016)** found a protein content of 21.82 ± 0.12% in the Large White breed, **Parunović et al. (2013)** in the Swedish Landrace 22.10%, **Ruusunen et al. (2012)** in various breeds in Scandinavia 21.8 to 22.9%, **Kameník, Saláková and Kašpar (2018)** in the Danish-Norwegian breed Topigs 24.40%, **Stanišić et al. (2015)** in Landrace 23.43 ±1.71%.

An important indicator which affects the taste properties of meat is the content of the intramuscular fat (IMF). According to **Fortin, Robertson and Tong (2005)**, the optimal IMF content in meat is 1.5 to 2.5%. The Ma breed had a higher IMF content (1.34 ±0.38%) compared to the LW breed (0.98 ±0.24%), but this difference was not statistically significant. Similarly, no significant differences were observed among groups with different slaughter weights. The IMF content was relatively low in both breeds compared to other authors, e.g. as far as the Ma breed is concerned**, Ender et al. (2002)** found the IMF content 9.00%, **Holló et al. (2003)** 8.38%, **Stanišić et al. (2015)** 6.40%. **Matoušek et al. (2016)** and **Nevrkla et al. (2017)** found the IMF content of the Czech autochthonous breed Prestice Black-pied pig at the level of 2.47% up to 2.89%. **Tomović et al. (2016)** found the IMF content at the level of 2.56% in the Large White breed.

Neither the breed nor the different slaughter weight had any effect on the cholesterol content in MLD. However, the Ma breed had by 4 mg.100g^{-1} of cholesterol in the meat more than the LW breed (44.17 ±5.77 mg.100g^{-1} vs.

Table 3 Correlation coefficients of the physico-chemical indicators *m. longissimus dorsi* in relation to slaughter weight and the IMF content.

	Slaughter weight	IMF
pH_1	0.304	0.086
pH_2	- 0.008	- 0.031
EC_1	0.160	- 0.038
EC_2	- 0.265	0.416
Drip loss	- 0.104	- 0.283
CIE L*	- 0.449*	- 0.282
CIE a*	0.598**	- 0.061
CIE b*	0.222	- 0.481*
Water	0.054	0.524*
Protein	0.356	- 0.506*
IMF	- 0.164	1
Cholesterol	- 0.159	0.813**

Note: IMT: intramuscular fat, *: p <0.05, **: p <0.01.

40.00 ±2.14 mg.100g^{-1}). Comparable values of the cholesterol content in MLD were also found by **Lípová et al. (2019)** in the Ma breed (43.01 mg.100g^{-1}) and the hybrids Ma x LW (38.91 mg.100g^{-1}).

Pârvu et al. (2012) have reported a similar cholesterol content in the Ma breed (41.64 mg.100g^{-1}), but they had a significantly higher cholesterol content of 61.24 mg.100g^{-1} in the LW breed. In contrast to our findings, other authors have reported higher cholesterol levels in MLD in the Ma breed, ranging from 61.14 to 62.90 mg.100g^{-1} **(Parunović et al., 2013; Petrović et al., 2010)**.

Table 3 shows the correlation coefficients of the physico-chemical indicators in relation to slaughter weight and the IMF content. The slaughter weight positively correlated with the indicator a* (p <0.01, r = 0.598) and negatively with the indicator L* (p <0.05, r = -0.449). It follows from the above that the pig meat is darker and redder with the increase of the slaughter weight. **Ba et al. (2019)** have stated that increase of the slaughter weight is associated with the redder and yellower meat. In terms of the physico-chemical indicators, the IMF content correlated negatively with the indicator b* (p <0.05, r = -0.481). In contrast, **Ba et al. (2019)** found a positive relationship between the value b* and the fat content (r = 0.652, p <0.05). Within the chemical meat composition, the IMF content correlated positively with water (p <0.05, r = 0.524) and cholesterol (p <0.01, r = 0.813) and negatively with the protein content (p <0.05, r = -0.506). Contrary to our results, several authors have found a negative relationship between the IMF content and water in the MLD muscle **(Tomović et al., 2014; Vranic et al., 2015; Lípová et al., 2019)**.

CONCLUSION

There were no major differences in the meat quality between the Mangalitsa and Large White breeds. Nevertheless, we can conclude that the breed Mangalitsa has showed more favorable values of the physico-chemical indicators, as it has achieved a significantly lower drip loss (7.57% vs. 10.13%, p <0.05), a lower value of L* (51.71 vs. 58.75, p <0.001) and a higher value of a* (3.52 vs. 0.59, p <0.001). That means that meat of the Mangalitsa breed had a better water holding capacity, was darker and redder in comparison to the Large White breed. Similarly, when comparing the quality of the meat with regard to the slaughter weight, there were no large differences between individual weight groups. However, a higher slaughter weight has positively influenced mainly the color of the meat, as pigs weighing more than 110 kg achieved a significantly lower value of L* (p <0.01) and a higher value of a* (p <0.001) in comparison to pigs of the lower weight. As a positive effect of a higher slaughter weight its effect on the protein content in the meat can be considered, as pigs weighing over 100 kg had a significantly higher protein content in the meat than pigs weighing below 100 kg (p <0.05).

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RESEARCH ON MILK HOMOGENIZATION IN THE STREAM HOMOGENIZER WITH SEPARATE CREAM FEEDING

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ABSTRACT

Homogenization, which is used in the technological schemes of production of most dairy products, is the most energy-intensive of the processes of mechanical processing of milk. One promising way to increase the energy efficiency of homogenization is to use separate homogenization and to use a little-researched stream homogenizer with separate cream feeding. The principle of its action is to pre-divide milk into cream and skim milk, and feed the fat phase with a thin stream into the stream of skim milk. This creates the conditions for achieving the high value of the Weber criterion – the main factor in the dispersion of milk fat. The purpose of these researches is to conduct experimental studies and determine the energy consumption and quality of homogenization of milk after treatment in a stream homogenizer. To achieve this goal, a designed experimental setup was used. The dispersive indices of the milk emulsion were determined by computer analysis of micrographs of milk samples obtained with an optical microscope and a digital camera using Microsoft Office Excel and Microsoft Visual Studio C # software using the OpenCV Sharp library. As a result of experimental studies, the critical value of the Weber criterion for homogenization of milk was determined, which is 28. The regularities of dispersion of milk fat in a stream homogenizer with separate feeding of the fat phase have been established. It is determined that the milk treatment in the experimental homogenizer allows us to achieve an emulsion with an average size of fat globules of about 0.8 μm (at the level of valve homogenizers). The value of the homogenization coefficient is obtained for the disruption of the fat globule in the conditions: subject to a single effect on the emulsion, without the influence of vibration and cavitation. This homogenization coefficient equals 3300 $m^{3/2}.s^{-1}$.

Keywords: milk; homogenization; homogenizer; stream homogenizers with separate cream feeding

INTRODUCTION

Today, the vast majority of milk as raw materials for the production of drinking milk, cream and other types of dairy products is homogenized **(Rayner and Dejmek, 2015)**. The main advantages of products manufactured using homogenization: providing uniformity of color, taste, fat content; improving the consistency, increasing the intensity of the white color, increasing the stability during storage, reducing the settling of fat and creating a fuller taste of the product.

But homogenization is one of the most energy-consuming processes in the vast majority of technological schemes for dairy production. The specific energy consumption of the most commonly used valve homogenizers reaches 8 $kW.h.t^{-1}$ and is the largest of the milk processing equipment **(Yong, Islam and Hasan, 2017a; Ashokkumar, Rink and Shestakov, 2011)**.

As a result of attempts to solve the existing disadvantages of homogenization, scientists have developed a wide range of homogenizers, such as valve, pulsation, vacuum, stream, ultrasonic, rotary, etc. However, none of them combines a high degree of grinding milk fat globules (such as valves) with low energy consumption **(Narvhus, Abrahamsen and Østlie, 2007; Yong, Islam and Hasan, 2017b)**.

The main reason for such problems is the lack of a unified theory and mechanism of dispersion of the fine-phase phase of fat emulsions and difficulties in obtaining experimental visual data of the process of disruption of fat particles of microscopic size.

Recent visual data on the process of fat phase dispersion in valve homogenizers confirm the validity of the turbulent viscous theory according to which the disruption occurs as a result of the destabilization of Kelvin – Helmholtz and Rayleigh – Taylor **(Håkansson et al., 2011; Håkansson et al., 2010; Innings and Trägårdh, 2005; Loitsyansky, 2003)**. Despite the significant differences between these hypotheses, they are common to create hydrodynamic

conditions in the fracture zone that contribute to the increase of the relative velocity of the emulsion phases. That is, the universal criterion for deformation and disruption of the fat globule is the Weber criterion (We), the main reason for dispersion being the difference in velocity between the fat globule and the surrounding plasma (relative velocity of phases or velocity of sliding of the fat globule) **(Håkansson et al., 2013)**.

The simplest and most obvious way to create a sliding fat globule is to separate milk fat from whole milk and feed it with a thin stream or film into the product's high-velocity stream of skim milk. This method is the basis for the construction of a stream homogenizer with separate cream feeding (SHSCF), in which the milk cream is fed by a thin stream perpendicular to the flow of skim milk (Figure 1) **(Deinychenko, Samoichuk and Kovalyov, 2016)**.

Additional intensification of the process of homogenization in such devices is due to the concentration of the supplied energy on the fatty phase of the emulsion using separate homogenization. Preliminary separation of milk into cream and skim milk and treatment of only the fat phase, which results in the reduction of the volume of the emulsion being processed, which leads to the proportional reduction of energy consumption **(Dhankhar, 2014; Samoichuk and Kovalyov, 2015)**.

Due to the selection of structural-technological and mode parameters it is possible to combine the normalization of milk fat content with the operation of homogenization.

Therefore, the purpose of the study is to determine the prospects of using a stream homogenizer with separate cream feeding for milk processing by experimentally determining the quality of homogenization and energy consumption.

Scientific hypothesis

The scientific hypothesis is the ability to solve the problem of high energy consumption for homogenization of milk by using a stream homogenizer with separate cream feeding. In such a homogenizer, when the fat fraction is fed into the stream of skim milk, a high difference of phase velocities is created between the dispersed and dispersive phases of the emulsion, which makes it possible to achieve higher values of the Weber criterion than in other types of homogenizers.

MATERIAL AND METHODOLOGY

Experimental equipment

For the experimental research of the SHSCF, an installation was developed, the scheme of which is presented in Figure 2 **(Samoichuk and Kovalyov, 2011; Samoichuk and Kovalyov, 2013)**.

Skim milk goes through the pipeline from the container 2 through the pump 1 into the homogenization chamber 4. From the tank for cream 12, with the pump 7 through the channel 11, the fat phase is supplied to the central zone of the homogenization chamber in the stream of skim milk, where the process of dispersion takes place.

To increase the flow of skim milk in the homogenization chamber, stream guides 5 made of stainless steel were installed, which are secured by hinges 10 and have adjustable rods to adjust the distance between the guides. The housing of the homogenization chamber is made of organic glass for process monitoring. A gauge 3 is required to control the fluid pressure values. In the container 2 there is an opening for draining the residues of the product. The finished product is poured into the container 8.

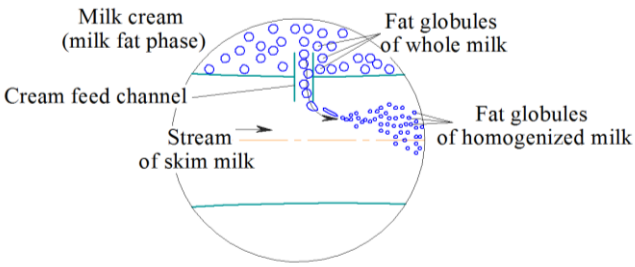

Figure 1 Scheme of homogenization in a stream apparatus with separate cream feeding.

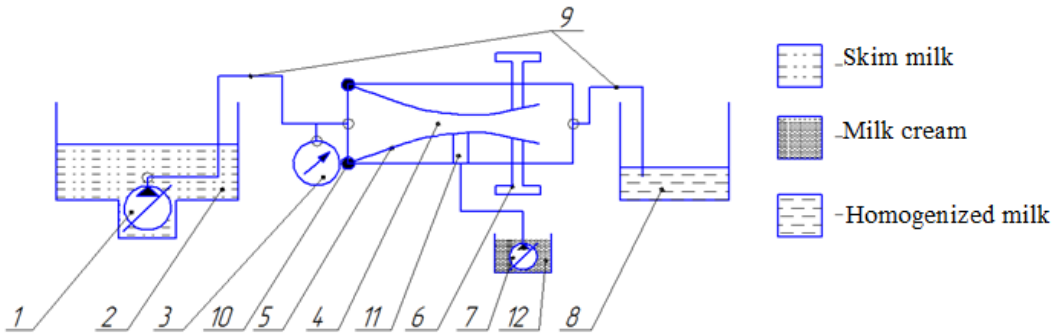

Figure 2 Scheme of the laboratory unit of the SHSCF. Note: 1 – rotor type pump; 2 – container for skim milk; 3 – pressure gauge; 4 – homogenization chamber; 5 – guides; 6 – adjusting rods; 7 – pump of supply of the fat phase; 8 – container for receiving the finished product; 9 – pipelines; 10 – hinges; 11– feed cream channel; 12 – tank for cream.

Prior to submission to the SHSCF, the milk is divided into skim milk and cream. Skimmed milk is fed under pressure at a certain rate, which increases in the central area of the device due to the narrowing of the flow, the value of which can be adjusted by rods. In the place of greatest narrowing, cream is supplied through a thin channel which diameter is 0.6 – 0.8 mm **(Samoichuk and Kovalyov, 2013; Samoichuk, Kovalyov and Sultanova, 2015)**. The channel of such a small diameter creates minimal flow resistance and allows the flow of cream with a thin stream. By varying the flow velocity in the cream feed zone, the distance from the end of the cream feed channel to the edge of the narrowing channel and the cream supply it is possible to investigate their effect on the quality and energy consumption of the milk fat dispersion process.

Figure 3 presents a general view of the laboratory unit for studying SHSCF.

In the central part of the chamber 3 (Figure 3), at the place of maximum narrowing, radial channels for feeding the fat phase are made.

Thus, thin streams of cream are fed into the high-velocity stream of skim milk, which creates the conditions for high-efficiency dispersion of milk fat – a high difference in velocity of phases (velocity of sliding the fat globule relative to the dispersion medium), which by Weber's criterion is the main parameter of the disruption of fat globules of milk.

The main parameter that determines the dispersion of the milk emulsion after treatment in SHSCF (Figure 4) is the relative velocity of the dispersed and dispersive phases, which is most influenced by the velocity of skim milk stream υ_{sm}, the velocity of the flow of cream υ_c, the diameter of the feed channel of cream d_c and its fat content F_c.

For the experimental studies, whole milk was used **(DSTU 8553:2015)**. Density 1027 – 1023 kg.m^{-3}, fat content 2.5 – 4.4%.

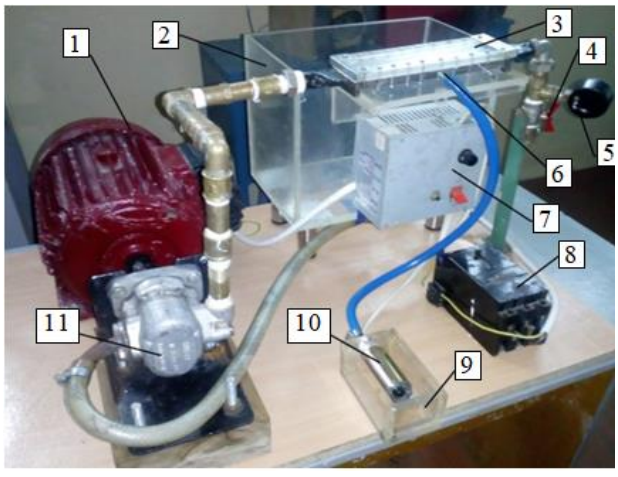

Figure 3 Structure of the laboratory unit for studying SHSCF. Note: 1 – electric motor; 2 – container for skim milk; 3 – homogenization chamber; 4 – throttle valve; 5 – pressure gauge; 6 – cream feed channel; 7 – control unit for the cream feed pump; 8 – electric starter of the main engine; 9 – tank for cream; 10 – pump for feeding cream; 11 – skim milk feed pump.

The average diameter of the fat globules of the emulsion (d), which should be provided as a result of homogenization, is 0.8 – 1.2 μm, which is sufficient for modern technological processes of milk processing.

The temperature of homogenization of milk was provided within the range of 60 – 65 °C. Numerous studies show that this temperature is optimal for the dispersion process. The minimum surface tension of the fat globule and the viscosity of the milk is ensured, the fat fractions go into the liquid state and no undesirable changes of properties occur under the action of high temperature **(Ion-Titapiccolo, Alexander and Corredig, 2013)**.

Statistical analysis

The dispersive indices of the milk emulsion were determined by computer analysis of micrographs of milk samples obtained with an optical microscope and a digital camera Mustek Wcam 300 (resolution 640 x 480). Each experiment was repeated 3 times. From each experiment, 3 samples were selected and 2 dilutions were prepared from each sample. 6 characteristic microscope field of view photos were selected from each dilution. Thus, 36 microscope fields of view were analyzed to determine statistical characteristics of milk.

The method of analysis of geometric characteristics of fat globules based on digital image analysis technologies was used to analyze the obtained micrographs.

The number of fat globules in the microscope field of view and their diameter were determined in the process of calculations. The average diameter of the fat globule was determined by the statistical method of power average (arithmetic mean).

For this purpose, the software module has been developed that is implemented in Microsoft Visual Studio 2013 based on C # using the OpenCV Sharp library set 4.2.0. The exported numerical data and the calculation of the sample statistics were performed in Microsoft Office Excel 2013. McBrain VA 318 electric wattmeter (Volga region plant of power equipment, Russia) was used to record power.

RESULTS AND DISCUSSION

Determination of the influence of the velocity of skim milk and the distance between the guides on the dispersion of milk emulsion

The main influential factor in the dispersion of the fat phase in the SHSCF is the rate of flow of skim milk, the change of which was varied by the supply of skim milk Q_{sm}. The results of the experimental studies and their comparison with the theoretical ones by the formula **(Samoichuk and Kovalyov, 2013; Samoichuk, 2018)** are shown in Figure 5.

As shown by the obtained data, the change in the distance between the guides (the area of intersection of the working chamber) has virtually no effect on the dispersion of the milk emulsion, which is consistent with the results of theoretical studies **(Samoichuk and Kovalyov, 2013; Samoichuk, 2018)**.

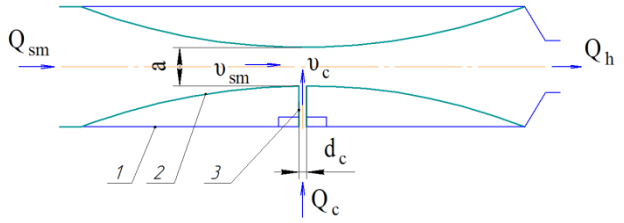

Figure 4 Calculation scheme of SHSCF. Note: 1 – chamber of stream homogenizer of milk; 2 – guides for the formation of a stream of skim milk; 3 – cream feed channel; a – distance between the guides; Q_{sm}, Q_c, Q_h – supply of skim milk, cream and productivity of SHSCF, $m^3.s^{-1}$.

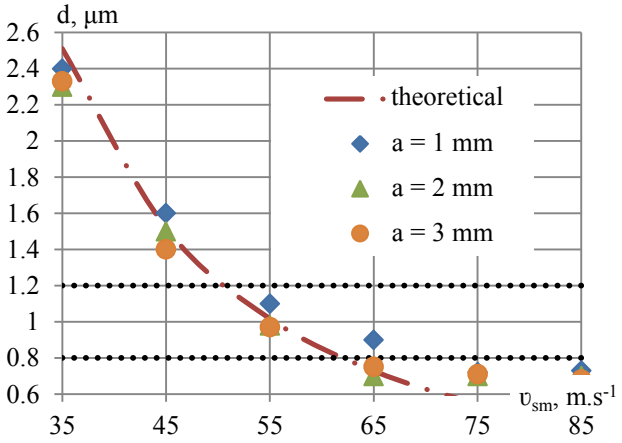

Figure 5 The dependence of the average diameter of fat globules d on the distance between the guides of SHSCF a and the velocity of skim milk υ_{sm} at d_c = 0.7 mm, υ_c = 80 $m.s^{-1}$, F_c = 30%.

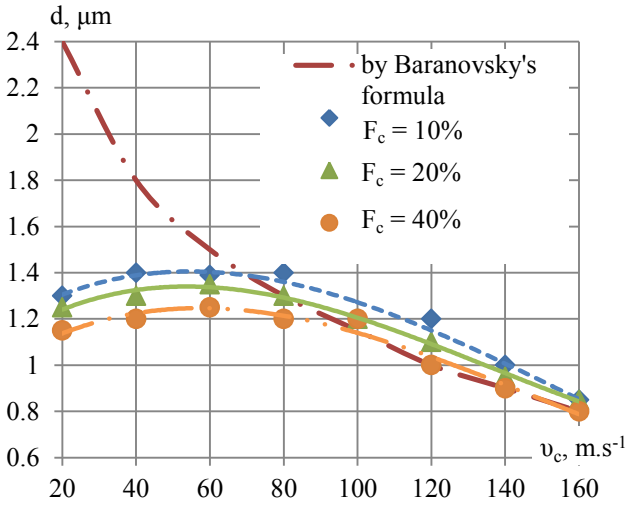

Figure 6 The dependence of the average diameter of fat globules ***d*** on the velocity of the flow of cream υ_c SHSCF and fat content F_c (at υ_{sm} = 60 $m.s^{-1}$).

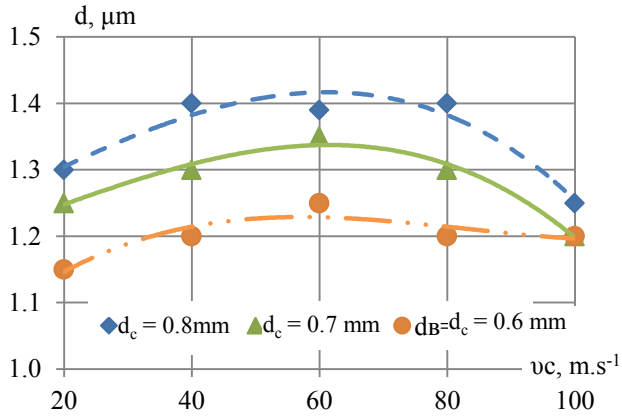

Figure 7 Dependence of the average diameter of the fat globules ***d*** on the velocity υ_c and the diameter of the feed channel of dairy cream d_c SHSCF (at υ_{sm} = 60 $m.s^{-1}$).

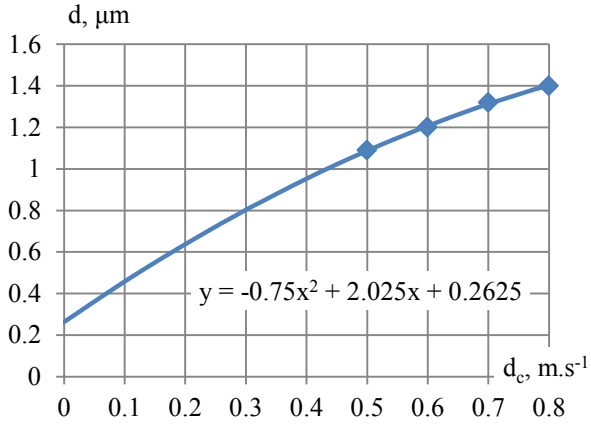

Figure 8 Prediction of the average size of fat globules of milk d for d_c <0.5 mm.

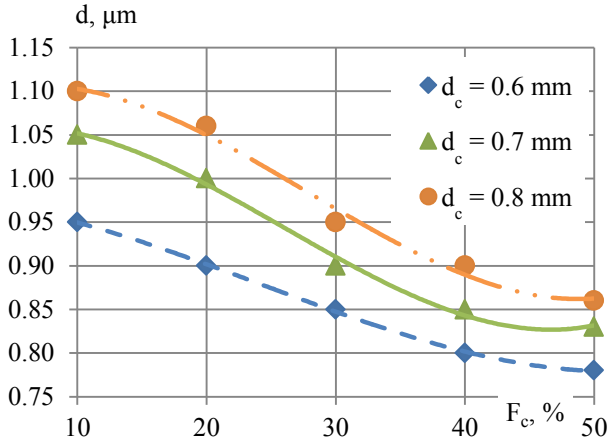

Figure 9 The dependence of the average diameter of the fat globules d on the diameter of the feed channel d_c SHSCF and cream fat F_c (at υ_{sm} = 80 $m.s^{-1}$).

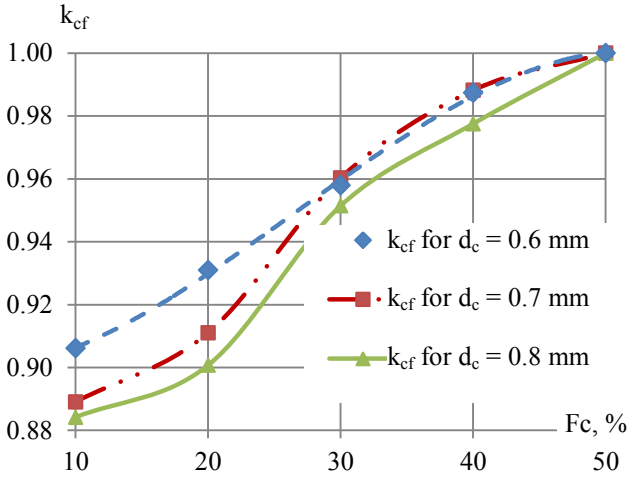

Figure 10 The dependence of the fat coefficient of cream for SHSCF k_{cf} on the fat content F_c, % (at υ_{sm} = 80 m.s^{-1}).

The slight increase in the size of the fat globules at a = 1 mm (by 2 – 5%) is explained by the increase in the Reynolds coefficient and the fluid turbulence. Increasing turbulence can be the cause of inefficient power dissipation, which is consistent with the results of the stream apparatus study **(Abiev, 2000)**. For SHSCF, the main factor in the disruption of fat globules is the sliding velocity of the fat globules, which increases as the flow rate of skim milk increases. To obtain an average size of fat globules of 0.8 μm, it is necessary to provide a skim milk velocity of 60 – 65 m.s^{-1}.

The experimental data are in good agreement with the theoretical data at critical Weber number We_k = 130, coefficient of SHSCF **(Samoichuk, 2018)** k_c = 0.7 in the range of 35 <υ_{sm} <70. However, the true critical Weber number can only be calculated after the experimental determination of the stream dispersion coefficient.

At a velocity of more than 70 m.s^{-1}, the dispersion is hardly increased. Similar is the graph of the dependence of the dispersion on the pressure for the valve **(Rovinsky, 1994; Loncin and Merson, 1979)**, pulsation homogenization with a vibrating rotor **(Samoichuk et al., 2016)** and flow-stream **(Samoichuk, 2018)** homogenization, which testifies to the similarity of the mechanisms of dispersion of fat globules in them.

Determination of effect of stream dispersion coefficient on average size of fat globules in milk

The use of higher fat cream increases the dispersion of the homogenized emulsion (Figure 6). This is due to the increase in the velocity of sliding fat globules of cream due to the decrease in the amount of plasma fed together with the cream. The dashed line shows the relationship between the size of the fat globules and the velocity of the cream in the disruption of the fat globules similar to the valve homogenization according to the formula by N. V. Baranovsky **(Rovinsky, 1994)**. If $\boldsymbol{v_c}$ >80 – 100 m.s^{-1}, the valve dispersion is the dominant principle of dispersion – the value of dispersion is close to that calculated by Baranovsky's formula.

In the range of 40 <$\boldsymbol{v_c}$ <80, the sizes of fat globules are maximal, and at $\boldsymbol{v_c}$ <30 m.s^{-1} they are reduced by 6 – 10% **(Samoichuk, 2018)**. The decrease in dispersion at the velocity of the stream of cream more than 30 m.s^{-1} can be explained by the presence of a steady stream of cream, which is destroyed only at the chamber wall which is opposite to the location of the channel of the cream. In this zone, the velocity of the skim milk stream is minimal, which results in lower values of the flow rate of fat globules.

If you do not take into account the dispersion of the type of valve homogenization, which is energy inefficient (in the range of 20 <$\boldsymbol{v_c}$ <80 m.s^{-1}), the highest degree of homogenization in SHSCF can be achieved at $\boldsymbol{v_c}$ <20 m.s^{-1}. By predicting with Excel spreadsheet tools at $\boldsymbol{v_c}$ <5 – 10 m.s^{-1}, the coefficient of influence of the flow velocity of the cream, SHSCF has a maximum value k_υ = 1.At 40 <$\boldsymbol{v_c}$ <80 k_υ =0.75 – 0.80, and at 20 <$\boldsymbol{v_c}$ <40 k_υ = 0.8 – 0.85.

Reducing the diameter of the cream channel leads to a decrease in the size of fat globules of milk (Figure 7).

This is due to the decrease in the central zone of the cream stream with a reduced flow rate of fat globules. Reducing the diameter of the channels from 0.8 to 0.6 mm leads to an increase in the dispersion by 8 – 10%. It is logical to assume that the maximum flow rate can be obtained by entering only one fat globule into the stream of skim milk. The maximum value of the coefficient of influence of the diameter of the channel of the fat phase will be at $\boldsymbol{d_c} = \boldsymbol{d}$. But testing this assumption in practice is difficult. The prediction made by Microsoft Excel (Figure 8) shows that at $\boldsymbol{d_c} = \boldsymbol{d}$ the average size of fat globules is 0.26 microns, therefore the coefficient of cream feed channel diameter k_{cd} = 1 at $\boldsymbol{d_c}$ = 0.26 mm.

Then the formula for the definition of k_{cd} looks like

$$k_{cd} = \sqrt{\frac{0.26}{2.025d_c - 0.75{d_c}^2 + 0.2625}}. \qquad (1)$$

Example: at d_c = 0.6 mm k_{cd} = 0.46; at $d_в$ = 0.7 mm k_{cd} = 0.44; at $d_в$ = 0.8 mm k_{cd} = 0.43.

The graph of the dependence of the dispersion of the emulsion on the fat content of the cream (Figure 9) indicates a decrease in the rate of decrease in the size of fat globules with increasing fat content of cream **(Samoichuk, 2018)**.

When the fat content of the cream is more than 40%, the dispersion of the emulsion is almost not reduced. The predicted minimum dispersion of milk is reached at F_c = 45 – 55%, where the coefficient k_{cf} = 1. For other values of the fat content of cream k_{cf} of the SHSCF are shown in the graph (Figure 10). In the range of F_c >30%, the values of the fat coefficient of stream dispersion do not differ for different d_c. At F_c <30% a significant effect is caused by the high turbulence of the flow and the mechanisms of disruption associated with the disruption of turbulent vortices, thus values of k_{cf} for d_c = 0.8 mm increase by 2 – 4% compared with d_c = 0.6 mm.

Therefore, to increase the degree of dispersion it is necessary to reduce the diameter of the feed channel of cream, use cream with a fat content of 30 – 50% and provide a feed rate of cream less than 30 – 40 m.s^{-1} or more than 80 – 100 m.s^{-1}.

Thus, for the data shown in Figure 6, the coefficient of stream dispersion will be equal (at k_{cd} = 0.44; k_υ = 0.75; k_{cf} = 0.96) k_c = 0.44 × 0.75 × 0.96 = 0.32.

The critical Weber number corresponding to the experimental data is $We_k = 28$. Compared to opposite-flow stream homogenization ($We_k^c = 500$ **(Samoichuk, 2008)**), this value is much smaller. But a direct comparison of these values is incorrect due to the use of the modified Weber criterion for opposite-flow stream homogenization – that is such We_k, where instead of the velocity of the sliding of the fat globule, the flow rate of the milk emulsion is used.

According to the obtained data of the critical value of the Weber criterion and **(Samoichuk, 2018; Samoichuk et al., 2019)**, the homogenization coefficient for SHSCF will be

$$K_h = \sqrt{\frac{28\ x\ 0.024\ x\ 0.1}{8\ x\ 980}\sqrt[3]{\frac{6}{3.14}}} = 3.300\ x\ 10^{-6}\ \mathrm{m^{3/2}.s^{-1}}.$$

The importance of the obtained results is that this value is obtained for the disruption of the fat globule in "pure" conditions: subject to a single effect on the emulsion, without the influence of vibration and cavitation. Therefore, the value of K_h is the largest among other types of homogenizers. For example, for the valve homogenizer, high turbulence and cavitation have a significant effect on the dispersion of milk fat, leading to a reduction in K_h to 1100×10^{-6} $m^{3/2}.s^{-1}$. For the pulsation homogenizer multiple treatments leads to a reduction in Kh to 225×10^{-6} $m^{3/2}.s^{-1}$ **(Deynichenko et al., 2018; Samoichuk, 2018; Samoichuk et al., 2019)**. For the pulsation homogenizer with a vibrating rotor, the influence of resonant phenomena, as well as the developed turbulence and cavitation in the gap between the rotor and the stator leads to a minimum $K_h = 68 \times 10^{-6}$ $m^{3/2}.s^{-1}$ **(Samoichuk et al., 2019)**.

CONCLUSION

Experimental studies of the dispersion of milk fat in SHSCF and the experimental determination of the stream dispersion index allowed us to determine the critical value of the Weber criterion for the disruption of the fat globule of milk in the milk plasma stream, which is 28. The value of the homogenization coefficient has been determined for the disruption of the fat globule under the condition of a single effect on the emulsion, without the influence of vibration and cavitation: $K_h = 3300 \times 10^{-6}$ $m^{3/2}.s^{-1}$.

On the basis of experimental studies of the patterns of dispersion of milk fat in SHSCF, it is established that to obtain an average size of fat globules of 0.8 μm it is necessary to provide a velocity of skim milk of 60 – 65 $m.s^{-1}$, to use cream, with a fat content of 40 – 45%, to ensure the velocity of the flow of cream 20 – 40 $m.s^{-1}$ and the diameter of the cream feed channel of 0.9 – 1.0 mm.

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COMPARISON OF HEAT-STABLE PEPTIDES USING A MULTIPLE-REACTION MONITORING METHOD TO IDENTIFY BEEF MUSCLE TISSUE

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ABSTRACT

Nowadays, proteomics is widely used as an analytical control method. A new method for determining animal tissue species-specificity based on a combination of two effective methods of food analysis, liquid chromatography (LC) and mass spectrometry (MS), was used in this work. Using this approach, it became possible to detect peptides. This work presents a comparison of species-specific, heat-stable peptides for the identification of beef. The objects of the study were native and boiled model mixtures containing beef with concentrations of 8% (w/w) and 16% (w/w). Pork was also added to the recipe to control for false-positive results. A high-performance liquid chromatography technique with mass spectrometric detection (LC-MS/MS) was used. Analysis of finished samples takes 25 minutes and is adapted to detect marker peptides. From the processing of the obtained data, three beef marker peptides were identified that were accepted as the best candidates. Two peptide prototypes, NDMAAQYK and YLEFISDAIIHVLHAK from the myoglobin protein and SNVSDAVAQSAR from the triosephosphate isomerase protein, were selected as potential biomarkers. For all samples, the signal-to-noise ratio (S/N) was set above 10. Temperature was not found to affect the structure and detection of marker peptides in samples with a muscle tissue concentration of 8% (w/w) at p <0.05. This approach is universally applicable for comparing biomarkers of other types of meat and to identify the most suitable candidates.

Keywords: biomarker; LC-MS/MS; prototype peptides; meat authentication; heat-stable peptide

INTRODUCTION

Over the past 15 years, extensive research has been conducted around the world on the study of protein substances in raw meat and meat products, both native and those formed in the process of various technological treatments.

A classic quantification method in proteomics is the use of an isotopic tag, the modification of which has more than 40 species **(Kopylov and Zgoda, 2007)**. There are also techniques that do not use isotopic labels **(Kopylov, Zgoda and Archakov, 2009)**. The sensitivity of protein determination compared with gel electrophoresis increases by several orders of magnitude. More recently, the complexity of the study of phosphorylated proteins has been overcome. Various post-translational modifications of proteins with high sensitivity and specificity are studied by the Selected Reaction Monitoring (SRM) method **(Zav'yalova, et al., 2014)**. Recently, a method of identifying species-specific molecular markers in the field of food analysis has gained strength, based on a combination of two methods, high-performance liquid chromatography (HPLC) and mass spectrometry (MS), used to detect peptides. Using this method, up to 0.5% (w/w) chicken meat was found in meat mixtures **(Sentandreu et al., 2010)**. In more recent studies, in boiled meat products, up to 1.0% (w/w) impurities of beef, pork, chicken, duck and goose were detected **(Montowska and Fornal, 2017)**. Heat treatment products were analysed using marker peptides derived from myosin 1 and 2 light chains. It is very important to determine the limit of detection (LOD) of the method. Using this criterion, one can compare various methods aimed at determining muscle tissue. Indicators of 0.5% and below were set for meat products. As an example, the established quantification limit for buffalo and sheep meat was up to 0.48% (w/w) meat **(Naveena et al., 2017)**. The good thermal stability of the peptides was demonstrated by the authors to identify horse and pork markers a lower limit of 0.24% **(Von Bargen, Brockmeyer and Humpf, 2014)**.

Scientific hypothesis

Using the S/N criterion, it is proposed that peptide markers be compared for the authenticity of raw meat and heat-treated meat. The aim of this work was to establish the best candidates for the species-specificity of beef. The selected biomarkers will be used for a highly specific and reliable method of multivariate identification and quantification of the proportion of muscle tissue.

MATERIAL AND METHODOLOGY

Model mixtures of minced muscle tissue were prepared in accordance with standard industrial procedures. A set of samples with a given recipe was prepared (Table 1). Beef muscle tissue content was 8% (w/w) and 16% (w/w). The calculation of muscle tissue content was carried out according to BEFFE (bindegewebseiweißfreies Fleischeiweiß − meat proteins that do not contain connective tissue) **(Leitsätze für Fleisch und Fleischerzeugnisse, 2016)**. Samples of minced meat mixtures were placed in a collagen shell and cooked to a core temperature of 72 °C.

Reagents and solvents

All reagents used were of U.S.P. purity or higher. All solvents, including water, were used with the LC-MS label.

Protein extraction

A 100.0 ±0.1 mg portion of each sample was weighed on an analytical balance (CP224S, Sartorius, Germany). A 1000 µL volume of denaturing buffer (6 M guanidine chloride) was added to the sample and ground in a mortar until completely dissolved. Samples of homogenized muscle tissue (MagNA Lyser, Roche Applied Science, Germany) were centrifuged at 10,000 rpm for 15 minutes at 4 °C (5430 R, Eppendorf, Germany) and 10 µL of sample was transferred to a 1.5 mL tube (for subsequent hydrolysis).

Protein digestion

Disulphide bridges were restored by adding 2 µL of dithiothreitol (0.5 M in water) and incubating the samples at 37 °C for 60 minutes (Thermomixer comfort, Eppendorf, Germany). Then, sulfhydryl groups were alkylated by adding 5 µL of iodoacetamide (0.5 M in water) and incubating them in the dark for 30 min at room temperature. Ultrafiltration at 13,000 rpm for 15 minutes at 4 °C using bicarbonate buffer was used to eliminate salts and denaturing agents. Protein content was measured by using a Quant-it protein analysis kit (Thermo Fisher Scientific, USA) with a Qubit fluorometer (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Trypsin digestion was carried out by using an enzyme-to-substrate ratio of 1:50 and incubating the reaction for 16 hours at 37 °C. Enzymatic hydrolysis was stopped by adding 1 µL of formic acid. Samples were stored at -20 °C and thawed before analysis.

LC-MS/MS analysis

For chromatographic analysis, a ZORBAX Eclipse Plus C18 column with a fast HD resolution of 2.7 µm (50 × 2.1 mm; Agilent Technologies, Santa Clara, California, USA) was used. Separation was performed by using an Agilent 1260 Infinity HPLC system (USA). The flow rate was set at 0.4 $mL.min^{-1}$, the column temperature was 30 °C, and the sample temperature was 19 °C; eluent A was water with 0.1% (v/v) formic acid, and eluent B was acetonitrile with 0.1% (v/v) formic acid. Gradient elution was performed as follows parameters: 0 min 95% A, 0 – 10 min from 95% A to 40% A, 10 – 15 min from 40% A to 0% A, 15 – 20 min 0% A, 20 – 21 min from 0% A to 95% A, 21 – 25 min 95% A (total analysis time 25 min). The injection volume was 10 µL for all types of samples.

Peptides were detected by using a three-quadrupole mass spectrometer (6410, Agilent Technologies, Santa Clara, California, USA) **(Khvostov et al., 2019)**.

Statistical analysis

STATISTICA 10.0 software was used in this study for statistical analysis. Significant differences were verified by using two-way analysis of variance (ANOVA), $p < 0.05$. Data were extracted from bioprograms in Microsoft Excel (USA).

RESULTS AND DISCUSSION

In this work, we used the **Skyline program (2019)**, capable of theoretically cleaving proteins and listing the SRM for each peptide (Table 2). Protein analysis was performed by using biomodelling. If it is necessary to process complete protein sequences during analysis of LC-MS/MS data, it is possible to use software such as Spectrum Mill (Agilent Technologies, Santa Clara, CA, USA) **(Sarah et al., 2016; Fornal and Montowska, 2019; Montowska and Fornal, 2017; Montowska and Fornal, 2019)**, Protein Lynx Global Server (Waters) **(Naveena et al., 2017)**, Peaks Studio software (Bioinformatics Solutions, Waterloo, ON, Canada) **(Prandi et al., 2017; Prandi et al., 2019)** and MASCOT (Matrix Science, Boston, MA, USA) **(Sentandreu et al., 2010; Naveena et al., 2017; Ruiz Orduna et al., 2015; Ruiz Orduna et al., 2017; Fornal and Montowska, 2019; Montowska and Fornal, 2017; Montowska and Fornal, 2019)**. In our work with the search for parameters for biomarkers on a mass spectrometer, the Skyline program proved to be the best. This is the best choice in the presence of a previously studied peptide sequence for develop of MRM methods. Most often, three transitions were selected. Only y-ions were used. The transition from parent ion (*m/z)* to product ions (*m/z)* occurred from a smaller to a larger one (*m/z)*.

Table 1 Muscle tissue content in the experimental mixtures.

Mixture	Beef (97% (w/w) muscle tissue), % (w/w)	Pork (90% (w/w) muscle tissue), % (w/w)	Pork (50% (w/w) muscle tissue), % (w/w)	Pork (20% (w/w) muscle tissue), % (w/w)	Total muscle tissue, % (w/w)
1	16.0	59.3	0.0	0.0	75.3
2	8.0	0.0	12.4	9.9	30.3
3	0.0	32.1	10.0	0.0	42.1

Table 2 Identification characteristics of beef (*Bos taurus*) heat-stable peptide markers for LC-MS/MS methods.

Protein	Marker peptide sequence	Parent ion (*m/z)*, product ions (*m/z)*	Collision energy (V)	Retention time (min ±*SD*)	References*
Myoglobin	HPSDFGADAQAAMSK	766.8 → 1395.6, 949.4, 892.4, 821.4	24.8	6.60 ±0.06	**Claydon et al. (2015); Li et al. (2018) Khvostov et al. (2019)**
		511.6 → 641.3, 635.3, 507.3	13.6		
	NDMAAQYK	470.7 → 580.3, 509.3	15.6	5.73 ±0.07	**Kulikovskii et al. (2019)**
	YLEFISDAIIHVLHAK	623.7 → 797.0, 732.4, 602.4	17.7	9.28 ±0.74	**Kulikovskii et al. (2019)**
Myosin-1	TLALLFSGPASGEAEGG PK	901.5 → 1290.6, 1143.5, 1056.5, 999.5 831.4	28.9	8.64 ±0.03	**Claydon et al. (2015); Fornal and Montowska (2019); Montowska and Fornal (2019)**
Myosin-2	MEIDDLASNVETISK	832.9 → 1061.6, 948.5, 877.5	26.8	8.26 ±0.01	**Montowska and Fornal (2019)**
	TLAFLFSGTPTGDSEAS GGTK	1022.5 → 1264.6, 1207.5, 1106.5	32.7	8.19 ±0.25	**Fornal and Montowska (2019)**
Myosin light chain 2f	EASGPINFTVFLNMFGE K	1001.0 → 1446.7, 1185.6, 985.5, 838.4	32.0	10.23 ±0.02	**Fornal and Montowska (2019)**
Stress-induced-phosphoprotein	ALDLDSNC[+57.0]K	518.2 → 851.4, 736.3, 623.2	17.1	7.71 ±0.92	**Wang et al. (2018)**
β-Hemoglobin	LHVDPENFK	549.8 → 848.4, 749.3, 634.3	18.0	7.08 ±0.17	**Li et al. (2018)**
Carbonic anhydrase 3	LVNELTEFAK	582.3 → 837.4, 708.4, 595.3	19.1	7.93 ±0.03	**Li et al. (2018)**
	GEFQLLLDALDK	681.4 → 1028.6, 900.5, 787.5	22.1	8.17 ±0.81	**Li et al. (2018)**
L-Lactate dehydrogenase A chain	DLADEVALVDVMEDK	831.4 → 1019.5, 948.5, 835.4	26.8	9.18 ±1.58	**Li et al. (2018)**
Triosephosphate isomerase	SNVSDAVAQSAR	602.8 → 904.5, 817.4, 702.4, 532.3	19.7	6.08±0.03	**Khvostov et al. (2019)**

Note: * Only the peptide sequence provided from the review article by **Stachniuk et al. (2019)**. The MRM transitions and Collision energy metrics were selected anew.

Peptides presented in a recent review **(Stachniuk et al., 2019)** were selected for comparison of potential biomarkers. Previously submitted peptides by us were analysed **(Khvostov et al., 2019; Kulikovskii et al., 2019)**. One of the criteria for marker specificity is the presence of a sequence of more than six amino acids **(Watson et al., 2015)**. This peptide length provides the species specificity of muscle protein. We decided to use the S/N indicator as the criterion for the comparison of heat-stable peptides.

Chromatograms of SRM peptide markers are shown in Figure 1a and Figure 1b. The four most intense peptides with a signal value of (50−250)*10^3 cps are presented in Figure 1a. The remaining peptides in the intensity range of (10 - 50)*10^3 cps are indicated in Figure 1b. The chromatogram data were obtained in a sample with a beef concentration of 16% (w/w), subjected to thermal treatment.

The S/N results for a sample of minced meat with 16% beef (w/w) after heat treatment are shown in Table 3.

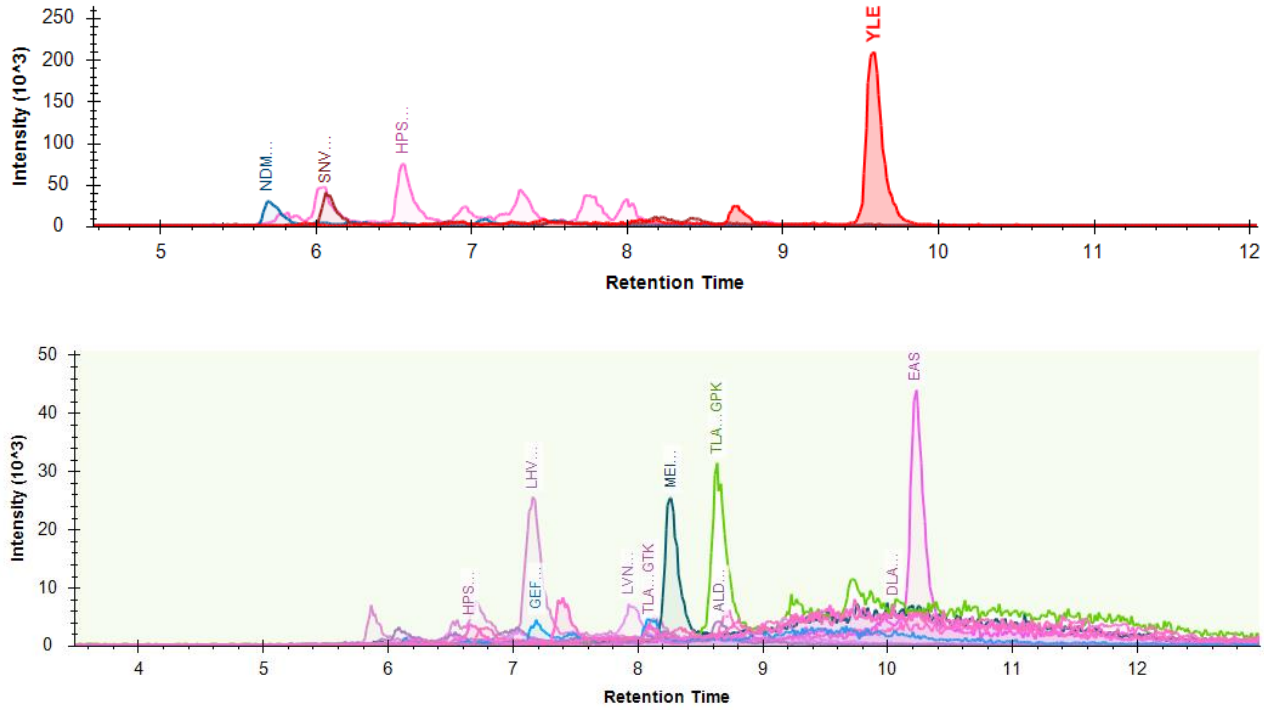

Figure 1 Chromatograms of selected biomarkers responsible for the identification of beef muscle tissue: major peptides (a) and minor peptides (b). Heat-treated mixture with 16% (w/w) beef.

The peptides are arranged in descending order of S/N. The data show that S/N is the highest for the peptide sequences NDMAAQYK **(Kulikovskii et al., 2019)** and YLEFISDAIIHVLHAK **(Khvostov et al., 2019)**, which are myoglobin derivatives. Since beef contains a high level of myoglobin, we obtained the largest number of myoglobin peptide derivatives. The S/N ratio is above 10 for both raw and heat-treated samples. For the peptide HPSDFGADAQAAMSK **(Claydon et al., 2015; Li et al., 2018; Khvostov et al., 2019)**, an additional MRM search was performed. Two parent ions, 766.8 (m/z) and 511.6 (m/z), were used. The most significant was ion 511.6 (m/z). The MRM intensity for this mass increased by 40% ±7.4 compared with ion 766.8 (m/z).

Samples were frozen and re-thawed. We evaluated the effect of one freeze/thaw cycle in digested samples on the intensity of the HPSDFGADAQAAMSK peptide in all mixtures. For samples subjected to and without heat treatment, S/N did not change. It was found that one freeze/thaw cycle did not affect the concentration of meat in mixture 1. If the beef content was less than 10% (w/w), the intensity decreased to 52.4 ±15.2. For peptides ALDLDSNC [+57.0] K **(Wang et al., 2018)**, DLADEVALVDVMEDK, and GEFQLLLDALDK **(Li et al., 2018)**, cross-contamination was recorded in a blank sample (no beef) (mixture 3) (Figure 2).

Many peptides did not meet the criterion of S/N >3.

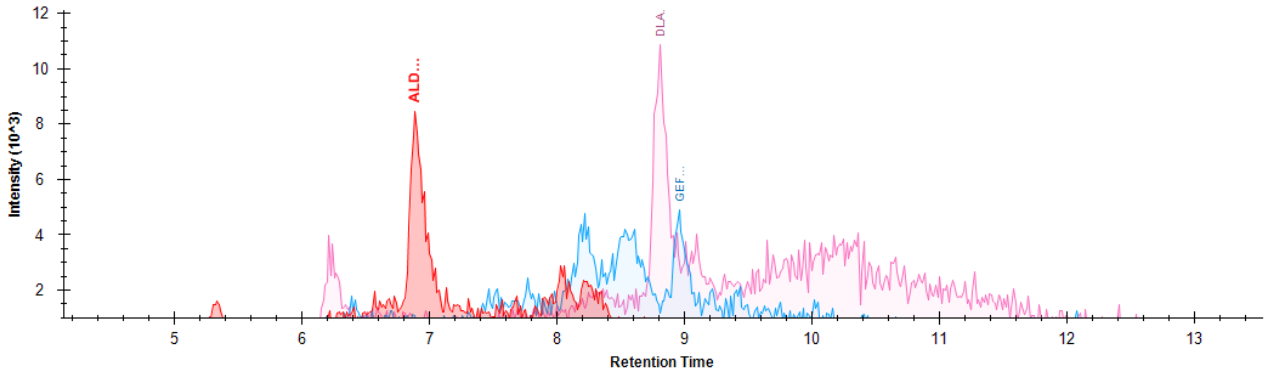

Figure 2 Peptides ALDLDSNC, DLADEVALVDVMEDK and GEFQLLLDALDK identified in samples not containing beef (mixture 3).

Table 3 Comparison of peptide markers with respect to signal-to-noise characteristics for two concentrations of beef muscle tissue and two cooking modes (without and with heat treatment).

Protein	Marker peptide sequence	Mixture 2 with beef 8% (w/w)		Mixture 1 with beef 16% (w/w)	
		not heated (S/N ±*SD*)	heat-treatment, (S/N ±*SD*)	not heated (S/N ±*SD*)	heat-treatment, (S/N ±*SD*)
Myoglobin	NDMAAQYK	12.50 ±2.45	24.61 ±4.82	11.53 ±2.64	127.66 ±12.51
Triosephosphate isomerase	SNVSDAVAQSAR	13.34 ±2.61	10.09 ±1.38	13.02 ±0.23	27.82 ±1.23
Myoglobin	YLEFISDAIIHVLHAK	3.24 ±0.64	7.79 ±0.76	4.64 ±1.97	24.06 ±7.58
	HPSDFGADAQAAMSK_511Freeze	1.36 ±0.27	2.14 ±0.50	1.95 ±0.17	7.78 ±0.42
Myosin-2	MEIDDLASNVETISK	2.47 ±0.48	2.29 ±0.18	3.5 ±0.42	8.33 ±0.79
Myosin-1	TLALLFSGPASGEAEGGPK	1.20 ±0.23	2.15 ±0.21	1.55 ±0.10	8.32 ±1.85
Myoglobin	HPSDFGADAQAAMSK_511	3.70 ±0.73	2.91 ±0.34	1.94 ±1.66	7.43 ±2.05
Stress-induced-phosphoprotein	ALDLDSNC[+57.0]K	2.51 ±0.49	1.74 ±0.51	2.32 ±1.07	4.66 ±0.76
β-Hemoglobin	LHVDPENFK	2.82 ±0.57	2.35 ±0.23	4.05 ±0.61	5.30 ±0.39
Myosin light chain 2f	EASGPINFTVFLNMFGEK	1.31 ±0.26	1.24 ±0.12	1.98 ±0.72	5.09 ±0.87
Myoglobin	HPSDFGADAQAAMSK_766	2.89 ±0.51	1.68 ±0.16	4.96 ±1.76	2.57 ±0.05
Carbonic anhydrase 3	LVNELTEFAK	1.04 ±0.26	0.60 ±0.08	1.78 ±0.48	2.54 ±0.24
	GEFQLLLDALDK	1.36 ±0.22	0.15 ±0.12	1.93 ±0.45	2.42 ±0.20
Myosin-2	TLAFLFSGTPTGDSEASGGTK	5.00 ±0.84	4.35 ±0.43	4.49 ±0.45	1.87 ±0.21
L-Lactate dehydrogenase A chain	DLADEVALVDVMEDK	0.2 ±0.12	1.36 ±0.13	0.58 ±0.13	0.51 ±0.35

Peptides representing from myosin proteins, such as MEIDDLASNVETISK **(Montowska and Fornal, 2019)** TLALLFSGPASGEAEGGPK **(Claydon et al., 2015; Fornal and Montowska, 2019; Montowska and Fornal, 2019)** were sensitive to heat-treated products with 16% muscle tissue (w/w). At lower concentrations, S/N approached 2 – 3. It was not possible to identify the DLADEVALVDVMEDK peptide **(Li et al., 2018)** in all types of samples. The S/N index for all samples was no greater than 1. A one-way analysis of variance (ANOVA) found an insignificant effect of temperature on the intensity of marker peptides at a concentration of 8% (w/w). In previous studies by **Kulikovskii et al. (2019)** and **Khvostov et al. (2019)**, we established a limit of detection (LOD) of 0.29% for the NDMAAQYK peptides and 0.93% for the SNVSDAVAQSAR peptide. From the analysis of species-specific marker peptides, three peptides for determining muscle tissue in beef were selected, taking into account the following factors: high prevalence in muscle tissues (>50 cps), good S/N ratio at low concentrations (S\N >10), high specificity and the presence of trypsin-specific cleavage sites at both ends of the protein chain.

Two-way analysis of variance does not reveal differences in the assessment of the criterion for the influence of heat treatment of mixtures at a concentration of 8% beef, confirmed by statistical calculation of p (<0.71), which is higher than the significance level of alpha (0.05).

CONCLUSION

The developed methodology allowed us to simultaneously identify and compare up to 13 beef peptide biomarker. Using the S/N criterion, it was possible to compare peptide markers for the authenticity of raw meat and heat-treated meat. Considered successful candidates whose signal-to-noise ratio was higher than 3.

From the analysis of species-specific marker peptides, three peptides for determining muscle tissue in beef were finally determined: NDMAAQYK and YLEFISDAIIHVLHAK from myoglobin and

SNVSDAVAQSAR from triosephosphate isomerase protein. For samples with two concentration levels and under cooking conditions at 100 °C, the S/N ratio was set above 10. This approach is universal. It is suitable for comparing meat biomarkers of other animal species. It will be able to identify the most suitable candidates. Selected peptide markers can be used to construct regression curves with good linearity, allowing a quantitative assessment of the types of meat present. The selected peptides can be used effectively to distinguish between accidental contamination (technologically unavoidable impurity) and deliberate falsification.

The developed methodology can aid in the study of the effect of meat protein on meat quality and functional characteristics, as well as the safety of finished meat products.

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RESEARCH OF QUALITY INDICATORS IN PROTEIN-BLUEBERRY CONCENTRATES

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ABSTRACT

The effect of blueberry coagulant in the form of specially processed blueberry paste on the building-up process of protein-blueberry concentrates has been considered in the article and the change in their physical-chemical indicators during storage has been determined. A study of blueberry coagulant application in the amount of up to 12% provides the effective running to process of thermo acid coagulation of milk proteins with a maximum clot yield (excluding limiting factors – organoleptic indicators and active acidity). It has been found that with an increase in the amount of blueberry paste adding from 2% to 10%, the yield of protein-blueberry concentrates increases from 5% to 40%, and the moisture mass fraction in clots decreases, on the contrary, from 73.4% to 67.1%. Other quality indicators of protein-blueberry concentrates were recorded: active acidity (from 5.0 pH at the beginning to 4.7 pH at the end of the storage life), and the water-retaining capacity at the level of (75.44 ±0.5%). Based on chromatographic studies, the degree of polyphenolic compounds transition (including anthocyanins) to protein-blueberry concentrates at the level of 52.26% has been determined by the calculation method and analyzed compared with control sample (blueberry paste) and colored whey. Based on the researches, protein-blueberry concentrates obtained by thermo acid coagulation of milk proteins are suggested to be used as basis in the cheese products recipes.

Keywords: coagulant; coagulation; concentrate; milk; protein

INTRODUCTION

Full use of all milk protein components during its processing is a condition for increasing the efficiency of obtaining concentrates. There are different methods of milk proteins coagulation: acid, acid rennet, rennet, thermo acid and thermo calcium. The most common methods include acid and rennet coagulation – the basics in the production of fermented milk product **(Bittante, Penasa and Cecchinato, 2012)**. According to the authors **(Kalmykova, 2013; Dabija and Sion, 2012)**, the production of protein products by acid milk coagulation is a popular and common practice. Enzymatic coagulation lasts until 10 hours. There is a need for the use of capacitive equipment. The technology of producing products by thermo acid coagulation of milk proteins involves less time, production space and equipment, but has several disadvantages **(Lyalin and Fedotov, 2009; Bittante, 2011)**. However, the degree of protein extraction during thermo acid coagulation is up to 95 – 97%, while for acid coagulation about 90%, and for rennet – 85% **(Osintsev et al., 2013; Ostroumova et al., 2009)**.

There is a problem of losing valuable proteins with whey in the production of milk protein clots. The use of various technological methods for the conversion of whey proteins to concentrate is actual. This approach will not only increase the yield, but also increase the biological value of the clots **(Abeykoon et al., 2016)**.

Complex precipitation of milk proteins can be achieved by thermo acid coagulation with various coagulants: acid whey (higher 150 °T) or food acids (hydrochloric, acetic, lactic, less often citric) **(Chinprahast, Subhimaros and Pattorn, 2015)**. Without limiting the foregoing, another task for the dairy industry is to increase the dairy products production with a high content of biologically active substances – using berry raw materials.

One of the qualitative characteristics of raw berry is acidity. Blueberries are different of variety and high content of organic acids at the level of (1.25 – 2.3 %). Organic acids at (93 – 95 %) represented by citric, malic, and in smaller quantities succinic, salicylic, and phosphoric **(Simakhina, 2018)**.

The biochemical composition of blueberries indicates not only high nutritional value, but also the pharmacological properties of this crop. It is expected that the therapeutic effect of blueberries is largely due to the content of phenolic compounds in it **(Vikul and Khomich, 2011)**. Berries have radioprotective properties, contribute to the neutralization of radionuclides in the body **(Van Breda, Briedé and de Kok, 2019)**.

For mitigate seasonal fluctuations in the use of berries in the dairy products production, including for coagulation, it

is advisable to attract raw materials, which are stable during storage. Because berries are products that quickly deteriorate, there is a need in their preservation to regulate biochemical processes **(Korotkiy, Korotkaya and Ibragimova, 2010)**.

A modern method of conservation is the processing of raw berry using cavitation devices and those that operate in the mode of developed turbulence **(Pakhomova, Dashkovsky and Stoyanova, 2012)**. Technologies of various homogenized berry pastes (blueberry, blackcurrant, lingonberry, etc.) of long-term storage with increased biological value have been deveped by using hydrodynamic cavitation processing of berry raw materials **(Bessarab et al., 2014)**.

Sterile pastes are industrially produced from blueberries with stable indicators, which excludes the introduction of extraneous microflora and, as a result, the production of a product safe by microbiological parameters during the storage period. Specially processed blueberry paste has an active acidity at 3.0 pH and the following chemical composition: dry soluble substances – 11.0%, polyphenols 457 mg.100g^{-1}, sugars – 7.92%, fiber – 1.57%, pectin – 0.27%.

Known technologies where berry raw materials are used as a filler in the production of cheese products, as well as a coagulant for the acid coagulation of milk proteins **(Shchetinin, Koltyugina and Kosynkina, 2011; Shchetinin et al., 2010)**. The development of milk-based products with berry raw materials is actual for using the functional and technological properties of berries and optimizing the composition of products in matter of vitamins and minerals. The use of berries as natural coagulants for thermo acid coagulation with the protein-berry concentrates production – the basis for cheese products, has not been enough investigated. The use of colored products as the basis for different dairy products will provide appropriate quality indicators with the exception of food colors and artificial flavorings, which corresponds to the concept of a healthy diet.

Scientific hypothesis

Scientific hypothesis is formulated, which is based on the assumption that it is possible to use blueberries as a coagulant, which will provide a clot yield, a natural color and the corresponding physicochemical parameters of the concentrates.

The aim of the work is to study the quality indicators of protein-blueberry concentrates obtained by thermo acid coagulation of milk proteins using blueberries in the form of a specially treated paste as a coagulant.

MATERIAL AND METHODOLOGY

The object of research is the quality indicators of protein-blueberry concentrates (PBC) obtained by thermo acid coagulation of milk proteins using blueberry paste as a coagulant.

Subjects of research – whole milk, blueberry paste (TU 10.8-2789021380-001 2012), yield, organoleptic, physical-chemical indicators (moisture mass fraction, active acidity, water-retaining capacity, polyphenol compounds content) of protein-blueberry concentrates.

The technology of protein-blueberry concentrates provides for thermo acid coagulation of milk proteins using berry raw materials as a coagulant according to the classical condition – coagulation temperature (93 – 95 °C) with duration of 5 min. To obtain a control sample (milk-protein concentrate), acid whey with titratable acidity of 160 °T in the amount of 8 – 10% of milk mass was used **(Grek and Skorchenko, 2009)**. The complex effect on milk proteins of high temperatures and acid reagents leads to the most complete coagulation of them. The coagulation process was established visually by the intensive formation of protein flakes and whey excretion. The obtained protein-blueberry concentrates were formed and self-pressed duration of (30 ±2 min). Pressing was carried out to a moisture mass fraction of 65 – 75%, depending on the further use of PBC for the manufacture of various cheese products.

In order to modify the method of thermo acid coagulation and rationalize the dose of blueberry coagulant (pH 2.6 ±0.2), a range from 2% to 12% with a variation step of 2% has determined. That particular amount in a different extent changed the active acidity in the mixture to ensure a balanced isoelectric state of milk proteins at a pH level (4.1 – 4.5) in the entire volume and led to their active coagulation **(Grek, Onopriichuk and Pshenychna, 2017)**. The products of this technological operation are protein-blueberry concentrates and the colored whey with a touch of blueberry coagulant added.

The yield (mass) of protein-blueberry concentrates was calculated by the gravimetric method based on the mass obtained from 4 dm^3 of milk and converted in percentage terms **(Pytel et al., 2017)**.

The active acidity was determined potentiometrically on Sartorius PB-20 universal pH meter **(Pytel et al., 2016)**.

The moisture mass fraction was investigated by the accelerated method on a KVARTS-21M-33 moisture meter and by the thermogravimetric method on an ADS series laboratory electronic moisture meter, manufactured by AXIS (Poland).

The water-retaining capacity (WRC) of protein-blueberry concentrates was determined by the Grau-Hamm method by A. A. Alekseev modification based on the determination of the water amount that is released from the product by light pressing, which is absorbed by filter paper **(Grek et al., 2015)**.

The polyphenol composition of protein-blueberry concentrates was determined by high-performance liquid chromatography using the spectrophotometer of Prominence LC-20 Shimadzu system (Japan). The substances identification in the extracts was determined by comparing the retention time and spectral characteristics of the investigated substances with similar characteristics of the standards in accordance with the method of polyphenols identification **(Mlček et al., 2019)**. Chromatography was performed at a wave-length of 225, 255, 286 and 350 nm **(Khodakov and Makarenko, 2010; Huang et al., 2012; Giovanelli and Buratti, 2009)**.

The titratable acidity of concentrates was determined according to **GOST 3624-92** and measured in degrees Turner (ºT). Turner degrees mean the amount of alkali solution milliliters of 0.1 mol.dm^{-3} that spent on neutralization of 5 g concentrate **(Grek et al., 2015)**.

Statistical analysis

The studies were repeated three times and processed mathematically using Microsoft Excel 2007 to provide accuracy of the obtained results.

RESULTS AND DISCUSSION

The obtained concentrates are a polydisperse colloidal system in which whey is the dispersion medium and milk proteins and dry substances of the blueberry coagulant are the dispersed phase. The yield and change in the moisture mass fraction of protein-blueberry concentrates depending on the amount of blueberry coagulant is shown in Figue 1.

The obtained yield results of the concentrates were corrected depending on the amount of dry substance of added blueberry coagulant. The research results (Figure 1) showed that under the same conditions of the thermo acid coagulation process with an increase in the amount of blueberry paste adding from 2% to 12%, the yield of PBC increases from 5% to 42%. The moisture mass fraction in PBC decreases, on the contrary, from 73.4% to 66.0%. Generally, the process is characterized by an increase in the transition degree of casein and the maximum amount of whey proteins into concentrates.

It has been established that with the addition of 12% blueberry coagulant, the yield of PBC increased by 2% compared with a sample containing 10% coagulant. The difference was in a margin of error, and the concentrate was characterized by too pronounced (berry) organoleptic indicators. Accordingly, for the further studies, the amount of blueberry coagulant has been selected at a level from 2% to 10%.

The next step of research was the study of changes in active acidity and water-retaining capacity of protein-blueberry concentrates for 72 hours at a temperature of (4 ±2 °C). The results are presented in the diagrams in Figure 2 and Figure 3.

Research has shown, that the active acidity of protein-blueberry concentrates depends on the pH of the blueberry coagulant and the amount of it added. So, the CBF sample, which was obtained by thermo acid coagulation of milk proteins with a blueberry coagulant in the amount of 10% (PBC10), has the lowest active pH value of 5.0 pH at the beginning and 4.7 pH at the end of the storage life. For control sample and all other PBC samples, the decrease in active acidity took place at (0.15 – 0.23 pH). This makes it possible to assert that the use of different amounts of blueberry coagulant for the production of PBC almost does not affect on this indicator of concentrates during storage. Similar results were reported by several authors **(Perreault et al., 2017).**

The more amount of blueberry coagulant (10%) used in thermo acid coagulation of milk proteins, the lower value of the water-retaining capacity in model samples of protein-blueberry concentrates – 71.36 ±0.5%. With a decrease in the amount of blueberry coagulant from 10% to 2%, the WRC of PBC increases to 8.14%. The average value of the water-retaining capacity of the obtained concentrates is (75.44 ±0.5%). During storage, a sharp decrease of the water-retaining capacity in all PBC is observed, and in the end the WRC ranged from 43.82% to 56.75%, which is on average 14% higher than in the control sample.

The dynamics of moisture evaporation from model samples on an Axis electronic moisture meter has determined to study the effect of blueberry coagulant on the qualitative and quantitative moisture state in concentrates. PBC sample obtained by thermo acid coagulation of milk proteins with a blueberry coagulant in amount of 6 ± 1% and a milk-protein concentrate obtained by classical technology were used for research **(Ondrušíková et al., 2019)**. The dynamics of moisture evaporation from concentrates is presented in Figure 4.

According to the measurement results, the main part of the moisture (free) has removed from the milk-protein concentrate (control) sample faster – in 18.0 ±0.5 min, and from protein-blueberry concentrate – more slowly and the indicator was within 21 ±0.5 min. There are differences in the speed of processes. During thermo acid coagulation, irreversible whey protein precipitation reactions occur with the loss of native properties, which is accompanied by the unfolding of the polypeptide chain of the protein molecule **(Donato and Guyomarc'h, 2009)**. As a result of such chain transformations and destruction of the tertiary and secondary structures, hydrophobic groups are "released" on the surface of the protein molecule. In this case, whey protein loses its solubility, aggregates and precipitates. It is likely that the interaction of milk proteins with carbohydrates of blueberry coagulant leads to the formation of additional complexes, which differ by the presence of strong bonds between pectins, dietary fiber and plasma proteins **(Chevalier et. al., 2019; Jakobek, 2015; Han et. al., 2011)**. So blueberries are carriers of vitamins, including vitamin C (14.1 – 26.4 mg.100g^{-1}), pectin substances (0.32 – 0.45%), phenolic substances (339 – 364 mg.100g^{-1})major mineral – and trace elements and other substances indispensable for the normal functioning of the body, with the ability to improve the consumer properties of products **(Toshev, Chaplinsky and Vakhitov, 2012; Li et. al., 2017)**. From a practical perspective, that is how you can justify using blueberry paste for thermo acid coagulation of milk proteins to bind free moisture and prevent syneresis.

PBC (presented in Figure 5) were characterized by the presence of the natural violet color inherent in blueberries, which contain anthocyanins, it was appropriate to study the degree of coloring substances transition to concentrates **(Mendelová et al., 2013)**. Coloring substances of raw berry are low molecular phenolic compounds, relate to bioflavonoids, in particular anthocyanins, which in plants are in the form of glycosides **(Medvecký et al., 2015).** It is known that the complex of blueberry phenolic compounds is represented by chlorogenic acid, kempferol and quercetin glycosides; free, condensed catechins and proanthocyanidins **(Aly, Maraei and El-Leel, 2019)**. The blueberry anthocyanin complex is determined by the set of main components: 3-glycosides and 3-rutinoside of dolphinidin and cyanidine, which is unchanged for berries of all varieties with black color **(Goldina, Safronova and Gaidul, 2015)**. In addition, the berries contain flavones, flavonols, catechins, hydroxycinnamic acids, which determine the natural violet color of the product **(Le Pham, 2020)**. The use of colored PBC as a basis for cheese products will ensure the exclusion of food grade dyes and artificial flavorings **(Slozhenkina et al., 2019)**.

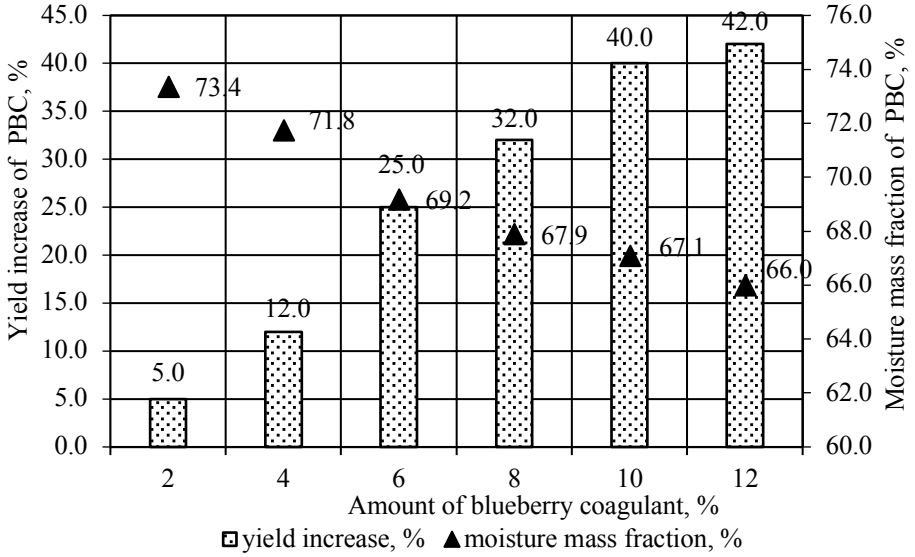

Figure 1 Yield and change in the moisture mass fraction of protein-blueberry concentrates on the amount of blueberry coagulant.

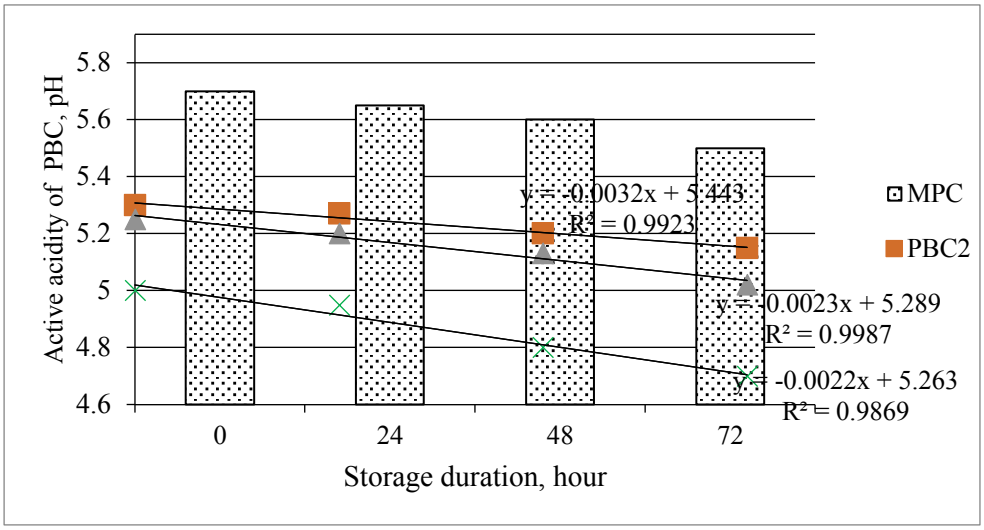

Figure 2 Change in active acidity of protein-blueberry concentrates.

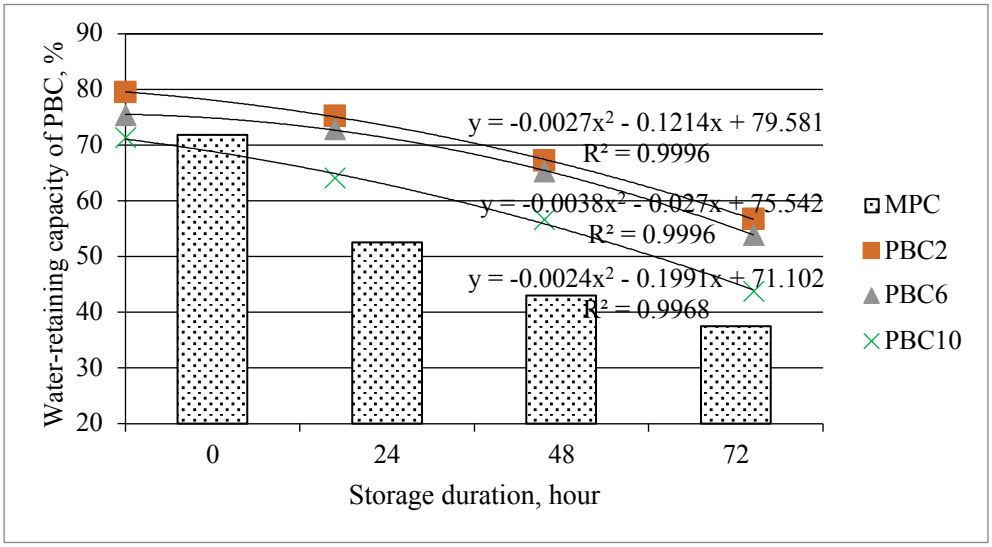

Figure 3 Change in the water-retaining capacity of protein-blueberry concentrates.

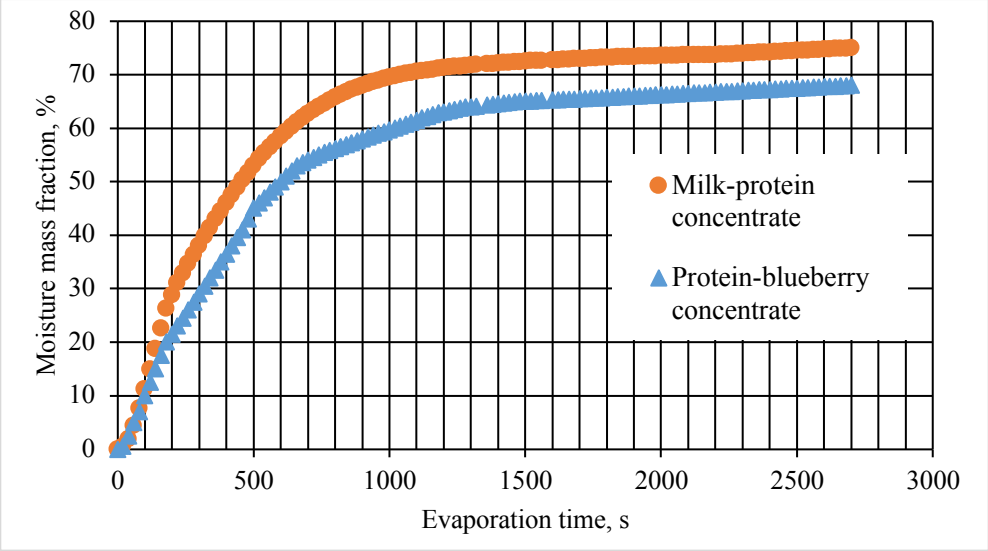

Figure 4 Dynamics of moisture evaporation from concentrates: milk-protein (control sample) and protein-blueberry.

Figure 5 Protein-blueberry concentrates obtained by thermo-acid coagulation of milk proteins by berry coagulant. Note: The amount of milk proteins by berry coagulant, %: a) 2; b) 6; c) 10.

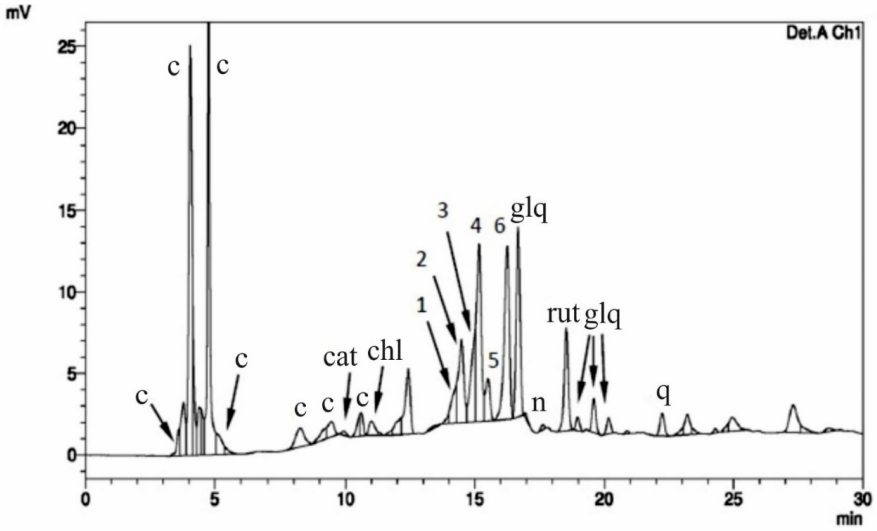

Figure 6 Chromatogram of protein-blueberry concentrates at 255 nm. Note: c – catechins, cat – catechin, chl – chlorogenic acid, n – naringin, rut – rutin, glq – quercetin glycosides, q – quercetin, 1 – delphinidin-galactoside, 2 – delphinidin-glucoside, 3 – cyanidin-galactoside, 4 – delphinide-arabinoside, 5 – cyanidin-glucoside, 6 – petunidine-galactoside + cyanidine-arabinoside + petunidine-glucoside + peonidin-galactoside.

The polyphenolic composition of blueberry paste (control sample), protein-blueberry concentrates and colored whey have been analyzed to determine the degree of coloring compounds transition. The chromatogram of protein-blueberry concentrates is presented in Figure 6.

Research results were analyzed in comparison with the control sample and the degree of their transition to separation products has been determined. The content of polyphenolic substances in protein-blueberry concentrates and colored whey is 281.10 and 146.19 mg.100g^{-1}, respectively. For comparison, the content of polyphenols in blackcurrant paste was on average 457 mg.100g^{-1}. The degree of polyphenolic compounds transition to PBC is 61.51% of their total number. About 31.99% of polyphenolic compounds, including anthocyanins, stay in colored whey. This effect is due to the weight loss correlation of the concentrate during technological operations, such as pressing and forming **(Cipolat-Gotet, et al., 2018)**.

Research results are fully sufficient to develop the technology of protein-berry concentrates obtained by thermo acid coagulation of milk proteins with a blueberry coagulant and subsequent use in the recipes of cheese products.

CONCLUSION

The quality indicators of protein-blueberry concentrates depending on the amount of blueberry paste added during the process of thermo-acid deposition of milk proteins have been studied. It has been found that with an increase in the amount of blueberry paste adding from 2% to 10%, the yield of protein-blueberry concentrates increases from 5% to 40%, and the moisture mass fraction in clots decreases, on the contrary, from 73.4% to 67.1%. Changes occur due to the complex coagulation and transition of milk proteins into a clot, as well as the hydrocarbon components of blueberry paste. This leads to the formation of additional complexes with tightly bound free moisture. The active acidity of protein-blueberry concentrates was in the range of 5.0 – 5.25 pH at the beginning of the storage life and decreased by (0.15 – 0.23 pH) for 72 hours. The average value of the water-retaining capacity of the obtained concentrates is (75.44 ±0.5%).

The degree of polyphenolic compounds transition (including anthocyanins) from berry raw materials in PBC at the level of 61.51% was determined by high performance liquid chromatography. About 31.99% of polyphenolic compounds, including anthocyanins, stay in colored whey of their total amount in blueberry paste.

Protein-blueberry concentrates can be used in the recipes of cheese products with a regulated moisture mass fraction, active acidity, water-retaining capacity and natural color.

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MASTITIS PATHOGENS AND SOMATIC CELL COUNT IN EWES MILK

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ABSTRACT

The aim of this study was to determine the occurrence of pathogens in selected group of ewes and the relationship between somatic cell count (SCC) and the presence of pathogens. The experiment was carried out on a dairy farm, where predominantly breed was a Tsigai. Sampling was carried out in monthly intervals as part of the milk recording test day from February to July 2019. A total of 303 ewes were included in the survey, during the milk recording test day. The ewes with SCC $\geq 1000 \times 10^3$ cells.mL^{-1} were selected for further sampling at half udder level. Based on SCC the ewes were divided into five groups: $<200 \times 10^3$; $\geq 200 < 400 \times 10^3$; $\geq 400 < 600 \times 10^3$; $\geq 600 < 1000 \times 10^3$; $\geq 1000 \times 10^3$ cells.mL^{-1}. The first group of SCC contained 33.9% of milk samples, the second 14.1% of samples, the third 5.7% of samples, the fourth 6.2% and the fifth 40.1% of samples. The most common pathogens were coagulase negative staphylococci (CNS). The most frequent CNS was *Staphylococcus (S.) simulans* (24.4%). *S. aureus* was identified in 5.3% of bacteriological positive samples. Almost 70% of ewes with bacteriological positive samples were repeated identified the presence of pathogens during tested period. SCC $\geq 500 \times 10^3$ cells.mL^{-1} were detected in 92.5% bacteriological positive milk samples. The presence of pathogens increased SCC in milk ($p < 0.001$) as compared to samples free of pathogens. In conclusion, the SCC $\geq 500 \times 10^3$ cells.mL^{-1} could be important for detection of subclinical mastitis at half udder level in dairy ewes.

Keywords: ewes; mastitis; somatic cell count; SCC; pathogens

INTRODUCTION

Mastitis is big healthy, economic and welfare problem in dairy animals. The main cause of increase SCC in milk of ewes is intramammary infection **(Souza et al., 2012)**. Thus, SCC in milk can be used as indicator for diagnostic of subclinical mastitis **(Tvarožková et al., 2019; Olechnowicz and Jaskowski, 2005; Leitner, Silanikove and Merin, 2008; Zigo et al., 2019)**. However, the physiological level of SCC in ewe's milk is still under discussion despite the researches. The results of researches point to the need to set a limit for physiological level of SCC in raw ewe's milk in relation to mastitis **(Persson et al., 2017)**. Our preliminary results also support that high SCC are caused by presence of pathogens **(Uhrinčať et al., 2019)**. CNS are the most common pathogens isolated from milk samples of ewes **(Holko et al., 2019; Zigo et al., 2014; Zafalon et al., 2018)**.

Scientific hypothesis

The hypothesis of this article is that high SCC in milk is caused by presence of mastitis pathogens. The aim of this study was the evaluation of occurrence of pathogens in milk of ewes and the possible relation of pathogens with SCC.

MATERIAL AND METHODOLOGY

The experiment was carried out on farm with Tsigai ewes as dominantly kept breed, together with Lacaune and Improved Valachian (303 dairy ewes in farm). The ewes were on pasture during the day and received concentrates in amounts of 200 g per day during milking.

The milk sampling was performed once a month during morning milking as a part of regular milk recording test day from February to July 2019 (February, March, May, June, July). SCC was determined using a Somacount 150 (Bentley Czech, USA). The ewes with SCC $\geq 1000 \times 10^3$ cells.mL^{-1} at any time during the regular sampling period were selected for further sampling at half udder level three days later. All these ewes were sampled again always on third day after further regular recording test days during whole lactation period even if they had low SCC at regular sampling. The milk samples were collected at half udder level and analysed on SCC and presence of pathogens. Thus 95 ewes (407 milk samples) without symptoms of clinical mastitis were selected into study.

For the bacteriological cultivation and the presence of pathogens the milk samples were colltableected by discarding first squirts of milk and subsequently cleaning of the teat end with 70% alcohol and approximately 5 mL of milk from each udder halves was taken in sterile tube.

The inoculum of each sample of milk was inoculated onto blood agar (Oxoid LTD, Hamshire, UK). All plates were incubated aerobically at 37 °C and evaluated after 24 hours. The all plates were re-evaluated after another 24 hours incubation. Colonies were identified on basis of cells morphology, Gram staining, type of hemolysis, the activities of catalase (3% H_2O_2, Merck, Darmstadt, Germany) esculin hydrolysis and cytochrome oxidase C (Bactident Oxidase, Merck). Presumptive *Staphylococcus aureus* were detected with the clumping factor test (DiaMondiaL Staph Plus Kit, Germany). Esculin positive streptococci were cultivated on modified Rambach agar to identification *Streptococcus uberis* or *Enterococcus* sp. according to **Watts, Salmon and Yancey (1993)**. Lancefield serotyping (DiaMondiaL Strept Kit, Germany) was used to characterize esculin negative streptococci. The species of gram-negative rods were identified used by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Contagious pathogens (*Staphylococcus aureus, Streptococcus agalactiae*) were classified as positive if one or more colony-forming unit (CFU) were found. Other pathogens were classified as positive if at least five CFU were found. Samples were classified as contaminated if three and more pathogens were isolated from one milk samples and growth of contagious pathogens were not identified.

The identification to species level by applying MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Briefly, fresh colony material was spotted by direct transfer method on to MALDI-TOF MS target plate, allowed to dry at room temperature and overlaid with 1 µL of matrix solution (saturated solution α-cyano-4-hydrosy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and allowed to dry at room temperature. Before the matrix solution was added 1 µL of 70% formic acid to the bacterial spot and allowed to dry for direct transfer-formic acid method. A loopful of bacterial colony was suspended in 300 µL distilled water and 900 µL ethanol was added for the protein extraction. The supernatant was discarded after centrifugation of cell suspension at 17,000 × g for 2 min. The centrifugation was repeated and the remaining ethanol was discarded. After dried the pellet was resuspended in 5 to 50 µL formic acid-water (70:30) in depending on the size of pellet and an equal volume of acetonitrile was added finally. 1 µL of the supernatant was transferred to the MALDI-TOF MS target plate after centrifugation at 17,000 × g for 2 min and allowed to dry before applying 1 µL of matrix solution. The MALDI Biotyper software version 2.0 (Bruker Daltonics) was used for bacterial identification.

Statistical analysis

Samples on the basis of SCC at half udder level were divided into following five SCC groups for evalutation of the distribution of milk samples: first $<200 \times 10^3$ cells.mL^{-1}; second $\geq 200 < 400 \times 10^3$ cells.mL^{-1}; third $\geq 400 < 600 \times 10^3$ cells.mL^{-1}; fourth $\geq 600 < 1000 \times 10^3$ cells.mL^{-1}; fifth $\geq 1000 \times 10^3$ cells.mL^{-1} (Using Excel, Microsoft, USA). The distribution of samples according SCC group was done by Microsoft Excel. Somatic cell score was used for statistical evaluation (SCS) and SCS was calculated according formula:

$$SCS = LOG_2 (SCC/100000) + 3$$

For statistical evaluation the data were divided according month of sampling where five groups of samples were created: Feburary, March, May, Juni and July. The samples also were divided into 9 pathogens group differed by presence of pathogens (1st group – major (*S. aureus, Str. agalactiae*), 2nd – minor (environmental pathogens other than CNS), 3rd – *S. simulans*, 4th – *S. schleiferi*, 5th – *S. caprae*, 6th – *S. epidermidis*, 7th – *S. chromogenes*, 8th – other CNS. Control group (9th group) consists from samples free of pathogens. Obtained data were processed by Microsoft Excel program and statistically evaluated by SAS/ 8.2 (2002). The model was tested by using Fisher's F-test. Differences between the levels of the effects were tested by Scheffe's multiple range test for studied trait. Data are presented as LSmeans ± standard error for evaluation of somatic cells the following model was used:

$$y = X\beta + Zu + e$$

y – was the measurements for somatic cell counts
β – the fixed effects of months, pathogens
e – random error, assuming $e \sim N(0, I\,\delta^2_e)$
X, Z – incidence matrices for fixed effects and random cow effect, resp.

RESULTS AND DISCUSSION

When evaluating the entire observation period tested ewes on udder half level the first group of SCC contained 33.9% of samples, the second 14.1% of samples, the third 5.7% of samples, the fourth 6.2% and the fifth 40.1% of samples (Figure 1). In our previous works we presented higher percentage (from 58.9% to 71.8%) of ewes in group $<200 \times 10^3$ cells.mL^{-1} under usual test day sampling for determination of physiological levels of SCC in healthy udder **(Tančin et al., 2017a; Tvarožková et al., 2018)**. The proposed physiological threshold of SCC for diagnosis of mastitis in Sarda sheep was determined by **Caboni et al. (2017)** at 265×10^3 cells.mL^{-1}. **Zafalon et al. (2016)** detected the value of SCC $>400 \times 10^3$ cells.mL^{-1} for diagnose of subclinical mastitis in flocks. Earlier proposed value of SCC for the diagnosis of mastitis was 500×10^3 cells.mL^{-1} **(Nunes et al., 2008)**. Low percentage of samples in group $<200 \times 10^3$ cells.mL^{-1} in present work could be explained by sampling schedule, where these samples were colected from the ewes with high SCC at udder level three days before. Thus some health problems of udder should be expected as it is presented later in article by cultivation of milk sample on pathogens presence. In ewes with SCC above 400×10^3 cells.mL^{-1} three or more months during lactation there were 5.6 to 7.5-fold higher probability of a subclinical mastitis in compared with ewes with SCC below above limit **(Spanu et al., 2011)**. **Berthelot et al. (2006)** reported 15% occurrence of subclinical mastitis if SCC in flock was over 650×10^3 cells.mL^{-1}.

Figure 1 Frequency of distribution of milk samples in SCC groups.

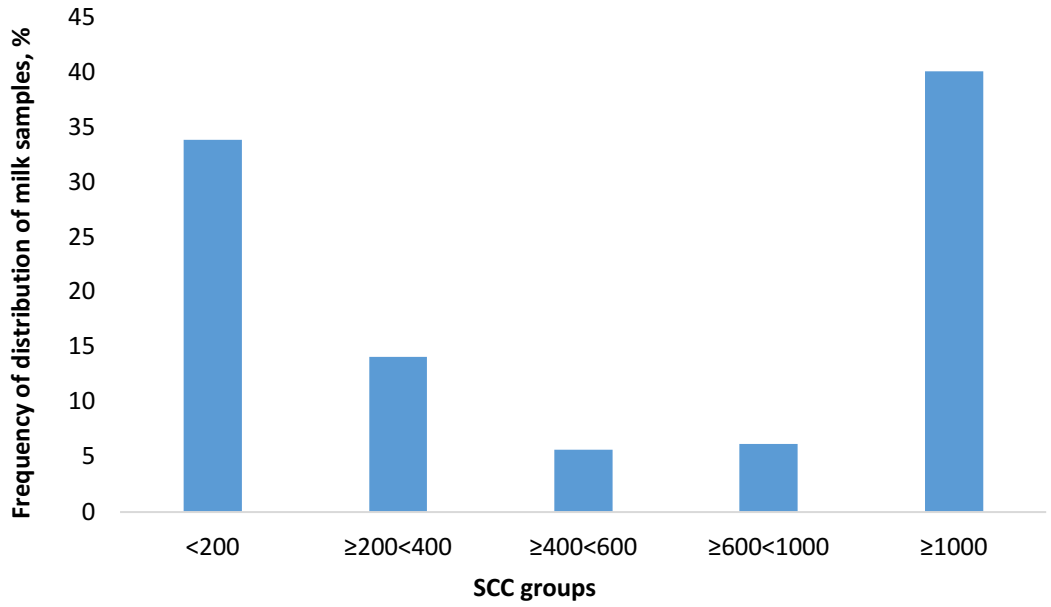

Table 1 The occurrence of pathogens in months of observation.

Pathogen	February	March	May	Jun	July
Enterococcus faecalis	2	1	3	-	-
Micrococcus luteus	-	-	-	-	1
S. aureus	1	2	4	1	-
S. capitis	1	2	-	-	-
S. caprae	2	3	2	1	20
S. cohni	-	-	-	-	1
S. epidermidis	3	7	3	3	5
S. felis	-	-	1	-	-
S. haemolyticus	1	-	-	-	-
S. chromogenes	5	4	1	-	-
S. lentus	-	-	-	-	1
S. piscifermentans	-	-	-	4	-
S. sciuri	1	-	-	-	-
S. schleiferi	-	-	8	21	-
S. simulans	7	6	15	3	2
S. warneri	-	-	1	-	-
Str. pluranimalium	1	1	-	-	-

Total 407 examined milk samples tested on presence of mastitis pathogens were as negative classified 63.1% of milk samples, out of these samples 75.9% had SCC below 500 × 10^3 cells.mL^{-1}. 36.1% of samples were classified as bacteriological positive and 0.7% of milk samples were classified as contaminated. Only 7.5% of bacteriological positive samples had SCC below 500 × 10^3 cells.mL^{-1}. Two pathogens were identified in 2.7% of bacteriological positive samples. In 67.9% of ewes with bacteriological positive samples there were repeated detected the presence of pathogens during tested period. Thus pathogens could persist in udder throughout whole lactation. Also up to 21.1% from these ewes had infected both udder halves pathogens repeatedly.

Important group of samples are those in fifth group – with very high SCC (Figure 1). In fifth SCC group there were almost 80% bacteriological positive samples, which indicated the reason of high SCC. Also even 92.5% bacteriological positive samples had SCC ≥500 × 10^3 cells.mL^{-1}. We detected significant lower SCS in milk of ewes without (4.03 ±0.12) than with the presence of any pathogens (p <0.001). There was no effect of different pathogens on SCS which ranged from 6.68 ±0.41 to 8.11 ±0.63. Also no effect of month of sampling on SCS was found out. Different pathogens could differently influence SCC **(Abu Baker Idriss et al., 2013; Bagnicka et al., 2011)**. **Kiossis et al. (2007)** used level of SCC of ≥500 × 10^3 cells.mL^{-1} and bacteriological positive milk samples for the diagnose of subclinical mastitis. Significantly higher SCC in bacteriological positive samples as compared with bacteriological negative samples found out **Ozenc et al. (2011)** in their study. **Świderek et al. (2016)** reported that milk samples without bacteria had the lowest average SCC. Also, **Persson et al. (2017)** detected significantly higher SCC for udder halves with intramammary infection compared to udder halves without bacterial findings.

From major pathogens only *S. aureus* was identified in 5.3% of bacteriological positive samples (Table 1). Other contagious pathogens were not found out in tested group of ewes. Low frequency of contagious pathogens in milk was also presented in our previous work **(Tančin et al. 2017b; Holko et al. 2018)** or abroad works **(Ergűn et al. 2009; Kern et al. 2013)**. **Zigo et al. (2011)** detected *S. aureus* in 9.3% of positive samples. **Moroni et al. (2007)** isolated *S. aureus* in 8.4% of infected milk samples. *S. aureus* was determined in 6.2% of subclinical mastitis cases **(Queiroga, 2017)**. The most frequent pathogens isolated from the milk samples were CNS. Also high occurrence of CNS was reported in study **Zigo et al. (2014)** and **Vasileiou et al. (2018)**. The most frequent CNS was *S. simulans* (24.6%) followed by *S. schleiferi* (21.6%), *S. caprae* (20.9%) (Table 1). From CNS found in farm had the highest occurrence *S. epidermidis* (36.3%) and *S. caprae* (21.3%) **(Pilipčincová et al., 2010)**. **Rahman et al. (2016)** showed that the most dominant CNS were *S. epidermidis*, *S. xylosus* and *S. chromogenes*. **Vasil' et al. (2018)** investigated that the most frequent CNS were *S. epidermidis* (24.3%), *S. schleiferi* (16.6%) and *S. chromogenes* (15.3%). *Enterococcus faecalis* and *Streptococcus (Str.) pluranimalium* was determined also (Table 1). **Zigo et al. (2017)** determined the incidence of *Enterococcus faecalis* in 6.1% of positive samples. **Grazia Puggioni et al. (2019)** detected 29.4% the occurrence of *Enterococcus faecalis* in their study.

CONCLUSION

CNS were the most common group of pathogens in milk followed by increased SCC in milk. *Staphylococcus (S.) simulans* (24.4%) was the most frequent CNS in milk samples. From contagious pathogens was identified *S. aureus* in 5.3% of bacteriological positive samples. The presence of mastitis pathogens during tested period were repeated detected in 67.9% of ewes with bacteriological positive samples. 92.5% bacteriological positive milk samples had SCC ≥500 × 10^3 cells.mL^{-1}. The high SCC ≥500 × 10^3 cells.mL^{-1} and bacteriological positive milk samples from udder halves may be useful criterion for detection of subclinical mastitis and poosible use for selecting ewes for dry treatment or culling.

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EFFECT OF THERMAL STABILIZATION ON PHYSICO-CHEMICAL PARAMETERS AND FUNCTIONAL PROPERTIES OF WHEAT BRAN

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ABSTRACT
The food industry also focuses on the use of by-products from food processing. Wheat bran is a valuable by-product of the wheat milling process, which is rich in dietary fiber. In addition to nutritional value, dietary fiber has a functional potential in the production of novel foods. Pre-treatment of the dietary fiber using different methods can change its functional properties. The objective of this study was to evaluate the effect of stabilization process on physico-chemical parameters and functional properties of wheat bran. Wheat bran from two wheat variety was treated using microwave and hot air heating. It was observed that wheat bran included more than 45% of total dietary fiber. Results suggested that treatment of bran using both method increased total dietary fiber content. Thermal treatment process decreased the anti-nutritional agent in bran samples. Phytic acid content diminishing of 44% and 49% was observed in microwave treated bran samples. Moreover, treatment of bran using a hot air heating improved the hydration properties (water holding, water retention and swelling capacity), while oil holding capacity was not significantly altered. Treatment decreased the antioxidant activity of treated bran samples. It was observed that thermal treatment modified the color parameters of bran (lightness, yellowness and hue angle decreased and redness and Chroma increased).

Keywords: wheat bran; thermal treatment; functional property, phytic acid; retention capacity

INTRODUCTION
With growing interest in health-promoting functional foods, the demand for natural bioactive additives has increased and the exploration for new sources is ongoing. The food processing industry in most countries generates large quantities of byproducts every year, which are frequently abandoned as wastes. However, many of these byproducts are dietary, functional, and potentially novel sources of nutrition. Of the many materials obtained, dietary fibers are particularly promising ingredients that has attracted considerable interest over the past few decades. The reason for this is their significant availability in most food byproducts, low costs, and positive effects for the prevention and treatment of a diverse range of diseases **(Han et al., 2017)**.

Dietary fiber is the edible part of plants or analogous carbohydrates; it consists of polysaccharides that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Total dietary fiber (TDF) is the sum of insoluble (IDF) and soluble dietary fiber (SDF) **(Lebesi and Tzia, 2011)**. The soluble and insoluble nature of dietary fibres involves differences in their technological functionality and physiological effects. SDFs are characterised by their capacity to increase viscosity, and to reduce the glycemic response and plasma cholesterol. IDFs are characterised by their porosity, their low density and by their ability to increase faecal bulk and decrease intestinal transit **(Elleuch et al., 2011)**. Compared with the IDF, SDF has superior beneficial properties for human health. In the natural plant cell walls, IDF accounts for a large proportion, while the proportion of SDF is very low. Thus, finding the appropriate method to convert more IDFs into SDFs is extremely important **(Gan et al., 2020)**. Thermal steps like roasting, steaming, cooking under forced air or microwave radiation usually have little impact on the chemical composition, but some effects on the processing properties could be observed **(Prückler et al., 2014)**.

There are various sources of dietary fiber. The most common source in bakery products is cereal bran, especially wheat bran **(Almeida, Chang and Steel, 2010)**. Thus, using wheat bran with high TDF content, fiber enrichment objectives can be achieved by means of small amounts of bran. The incorporation of lower levels of bran means a less negative impact on the finished product quality **(Ellouze-Ghorbel et al., 2010)**. Nutritionally, bran fractions produced by milling are rich in fibre, minerals, vitamin B6, thiamine, folate and vitamin E and some phytochemicals, in particular antioxidants such as phenolic compounds. However, bioavailability is affected by the

food matrix as well as processing conditions. Bran is used in the production of brown and wholemeal flours, hence retaining some of the valuable nutritional components that are depleted when these fractions are further removed in the refinement of white flour **(Stevenson et al., 2012)**. Wheat bran is therefore composed of pericarp, seed coats, and aleurone layer with some attached remnants of endosperm. Considerable amounts of wheat bran are produced annually that are mostly used in animal feeding. Due to the presence of the aleurone layer, wheat bran constitutes, however, a potential source of micronutrients that could be better valorized in human nutrition **(Antoine et al., 2003)**. The physiological effects of wheat bran can be split into nutritional effects (from the nutrients present), mechanical effects (mainly on the gastrointestinal tract, due to the fibre content) and antioxidant effects (arising from the phytonutrients present such as phenolic acid and alkylresorcinols) **(Stevenson et al., 2012)**.

Scientific hypothesis

This work evaluated the effect of different treatment process (hot air heating and microwave heating) on the physico-chemical parameters and functional properties of treated wheat bran.

MATERIAL AND METHODOLOGY

Material

Wheat bran from the variety PS Bertold (BB) and PS 215 (WB) were observed from Research and Breeding Station, Vígľaš Pstruša, Slovakia and Research Institute of Plant Production Piešťany, Slovakia. Wheat bran samples were treated using hot-air and microwave heating acording to method described by **Lauková et al. (2019)**.

Chemical composition

Chemical composition of wheat bran included determination of: moisture (AACC Method 44-19.01), ash (AACC Method 08-01.01), protein (AACC Method 46-13.01) and crude fat (AACC Method 30-25.01) **(AACC, 2000)**.

TDF, IDF and SDF content was determined by enzymatic-gravimetric method **(AOAC, 2003)**.

Phytic acid content was measured using colorimetric method according to **McKie and McCleary (2016)**.

Antioxidant activity of wheat bran was determined according to the method of **Cai et al. (2014)** by measuring free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity. Wheat bran (0.1 g) was extracted with 1 cm^3 of pure methanol at 25 °C for 2 h with continuous shaking under a dark environment and centrifuged at 1,200 × g for 10 min. The extract (0.05 cm^3) was reacted with 1 cm^3 of 0.1 mM DPPH solution at 25 °C for 30 min, and absorbance was measured at 517 nm. Antioxidant activity was calculated as percent discoloration of DPPH = $[1 – (A_1/A_0)] \times 100$, where A_1 is the absorbance of wheat bran extract at the end of the reaction (t = 30 min) and A_0 is the absorbance of the pure methanol control at the beginning of the reaction (t = 0). The data were reported as percentage of discoloration.

Functional properties

Swelling capacity, water absorption and water retention capacity were determined according to method described by **Lauková et al. (2018)**. Oil holding capacity was evaluated using method presented by **Mora et al. (2013)**.

Solvent retention capacity (SRC) tests were performed according to the method from authors **Xiao et al. (2006)** using 5% lactic acid, 5% sodium carbonate, 50% sucrose, and distilled water. Sample (5g) was added into a 50 cm^3 centrifuge tube with a screw cap. Then 25.0 cm^3 of an appropriate solvent was added and the mixture was vortexed vigorously to suspend the flour for 5 sec. The mixture was allowed to set and swell for 20 min and was vortexed for 5 s each at 5, 10, 15, and 20 min. After centrifugation at 1,000 x g for 15 min (not including time to achieve speed), the supernatant was decanted and the tube was drained at a 90° angle for 10 min on a paper towel. Finally, SRC value (14% mb) was calculated for each solvent as:

$$SRC\ (\%) = \left(\frac{gel\ weight - flour\ weight}{flour\ weight}\right) \times 100$$

Color of wheat bran

The color parameters *L* (lightness), *a* (redness/greenness), and *b* (yellowness/blueness) of the samples were measured by spectrophotometer Cary 300 UV-Vis (Agilent Technologies, Santa Clara, USA) with DRA-CA-30I sphere accessory. The spectrophotometer was calibrated with a white calibration tile. The Cary WinUV software with "Color" application was used for recalculation the Chroma (*C*) and hue angle (*H*). Color coordinates were determined five times per bran sample.

Statistical analysis

All determinations were carried out in triplicate unless otherwise state. The results were expressed as mean ± standard deviation. The significant differences between mean values of raw and treated bran were establish using a Student`s test at $p < 0.05$. The XLSTAT statistic software was used for data evaluation.

RESULTS AND DISCUSSION

Chemical composition

Composition of wheat bran is purely based on the variety, cultivation conditions and the methods employed for its separation, which determines the amount of starch attached to the aleurone layer after the separation **(Babu et al., 2018)**. Chemical composition of treated and raw wheat bran is summarized in Table 1. Results presented that moisture content of bran decreased after thermal treatment, which was caused by loss of water as a result of heating **(Dong et al., 2019)**. Furthermore, it was observed that treatment of wheat bran using microwave and hot air heating had no significant effect on ash and fat content.

Wheat bran contains more than 15% high quality proteins, but most of them are enclosed within a matrix of cell wall polysaccharides and so they are poorly digested. Wheat bran proteins have also been explored as a source of amino acids and bioactive peptides or as inhibitors of enzymes of industrial interest **(Balandrán-Quintana, Mercado-Ruiz and Mendoza-Wilson, 2015)**. From the

results concluded that protein content of raw bran varies from 15.46% (WB) to 15.92% (BB). These results were in agreement with those obtained by **Ferreira, Chang and Steel (2011)** (15.30%) and **Noort et al. (2010)** (15.90%). After microwave treatment, the protein content increased up to 16.56% (WB) and 17.20% (BB).

Wheat bran appears as an important dietary fiber source **(Ferreira, Chang and Steel, 2011)**. It was observed, that TDF content of raw bran samples were 46.35 and 45.99%, indicating bran as good source of dietary fiber. Similar TDF content in wheat bran was found by **Ma, Lee and Baik (2018)** (46.50%) and **Ferreira, Chang and Steel (2011)** (46.30%). Furthermore, IDF and SDF content of raw BB, 44.93% and 1.42%, respectively, were higher compared to raw WB (44.62 and 1.37%). Thermal treatments can change their physico-chemical properties of dietary fiber by altering the ratio between soluble and insoluble fiber (SDF/IDF), TDF content **(Ozyurt and Ötles, 2016)**. It can be concluded, that treatment of bran using both methods had significant effect on TDF and IDF. The results suggested that hot air treatment resulted in higher fiber content compared to microwave treated bran. Moreover, hot air treatment significantly increased the SDF content. In general, the changes in the dietary fiber composition during thermal processing may be partly attributed to the redistribution of the insoluble and soluble components of dietary fiber, and partly to the formation of resistant starch. An increased temperature breaks week bonds between polysaccharide chains and split glycosidic linkages in the polysaccharides **(Căprită, Căprită and Hărmănescu, 2012)**. Increase in total fiber can be also attributed to the formation of fiber-protein complexes that are resistant to heating and are quantified as dietary fiber **(Dhingra et al., 2012)**.

Bran also contains phytic acid which is the major phosphorus storage component and comprises 80% of total phosphorus in cereal grains **(Aktas-Akyildiz et al., 2017)**. The presence of phytate has been considered as an anti-nutrient in humans because of its effect on the bioavailability of iron, magnesium, zinc and calcium. While the mechanism is not entirely understood, it is suggested that phytic acid binds strongly with these mineral cations to form phytate-mineral complexes, changing their solubility, functionality absorption and digestibility. Consequently, the complex cannot be absorbed or easily hydrolysed by the human body and so there is an adverse effect on bioavailability of minerals **(Stevenson et al., 2012)**. The phytic acid content (Figure 1) in raw WB samples was higher (51.9 $mg.g^{-1}$) than in raw BB (40.4 $mg.g^{-1}$). These values are in agreement of those reported by **Noort et al. (2010)** (47.9 $mg.g^{-1}$) in wheat bran. It can be stated that thermal treatment significantly decreased phytic acid content in both bran variety. After microwave treatment, the phytic acid content was significantly reduced to 22.6 $mg.g^{-1}$ (BB) and 26.4 $mg.g^{-1}$ (WB). Recently, **Mosharraf, Kadivar and Shahedi (2009)**, and **Zhao, Guo and Zhu (2017)** also described a decrease in phytic acid content in treated wheat bran after steeping in acetate buffer and fermentation.

The antioxidant activity of wheat bran measured using DPPH is illustrated in Figure 2. The results showed that antioxidant activity of raw wheat bran was 24.84% and 28.85% for BB and WB, respectively. Both values were higher than those reported by **Verma, Hucl and Chibbar (2008)** (12.5 – 20.1%) in bran from 51 wheat cultivars. On the other hand, **Cai et al. (2014)** observed higher antioxidant activity (29.2 – 53.6%) in bran from American and Korean wheat varieties. Microwave and hot air heating of bran decreased its antioxidant activity. The lowest antioxidant activity values were recorded after hot air heating of bran samples (23.11 and 23.64% for BB and WB).

Functional properties

The technological interest and physiological effects of dietary fibre are related to their functional properties. The hydration properties of dietary fibres determine their optimal usage levels in foods because a desirable texture should be retained **(Yaich et al., 2015).** Wheat bran is rich in polysaccharides which can bind water on a molecular level through formation of hydrogen bridges. These mechanisms contribute to water uptake by bran in the case of unconstrained hydration. Alternatively, when bran is exposed to an external stress, only the water strongly bound in nanopores or through hydrogen bonds will govern water retention **(Hemdane et al., 2016)**. Functional properties of untreated and treated wheat bran are summarized in Table 2.

WHC is related to the porous matrix structure formed by polysaccharide chains which can hold large amounts of water through hydrogen bonding **(Du, Zhu and Xu, 2014)**. The WHC of raw bran samples were 2.46 $g.g^{-1}$ (BB) and 2.57 $g.g^{-1}$ (WB), which was similar to those described by **Cai et al. (2014)** (2.04 – 2.51 $g.g^{-1}$). From the results concluded that thermal tretment of bran resulted in increased WHC values. Moreover, hot air treated bran had higher WHC than microwave treated bran. The high WHC of modified bran suggested that this material could be used as a functional ingredient to avoid syneresis and to modify the viscosity and texture of formulated products in addition to reducing calories by the total or partial substitution of high-energy ingredients **(Grigelmo-Miguel, Gorinstein and Martín-Belloso, 1999)**. **Yan, Ye and Chen (2015)** demonstrated higher WHC value of bran after extrusion.

WRC is one of the major key parameter which has been studied in functional food. Most significant changes that happen during baking i.e. gelatinization of starch, denaturation of protein, flavor and color formation are due to water contents **(Khan et al., 2018)**. WRC is related to the content of insoluble dietary fiber and the intact cell structure of bran **(Zhao, Guo and Zhu, 2017)**. Raw BB bran had lower WRC value (1.74 $g.g^{-1}$) compared to WRC value of raw WB bran (2.16 $g.g^{-1}$). This WRC values are in agreement with result (2.18 $g.g^{-1}$) presented by **Ma, Lee and Baik (2018)**. After hot air treatment the WRC significantly increased up to 2.38 and 2.63 $g.g^{-1}$ for BB and WB, respectively.

Table 1 Chemical composition of wheat bran.

	Moisture (%)	Protein (%)	Ash (%)	Fat (%)	TDF (%)	SDF (%)	IDF (%)
Raw bran							
BB	7.92 ±0.09	15.92 ±0.11	2.89 ±0.07	2.91 ±0.03	46.35 ±0.03	1.42 ±0.01	44.93 ±0.03
WB	8.37 ±0.06	15.46 ±0.09	2.90 ±0.03	2.39 ±0.02	45.99 ±0.08	1.37 ±0.06	44.62 ±0.03
Hot air treated bran							
BB	3.18 ±0.06*	16.19 ±0.06*	3.30 ±0.01	2.10 ±0.02	49.74 ±0.05*	2.68 ±0.06*	47.06 ±0.02*
WB	2.84 ±0.06*	16.36 ±0.11*	2.84 ±0.11	2.19 ±0.03	47.80 ±0.06*	2.06 ±0.01*	45.74 ±0.05*
Microwave treated bran							
BB	3.51 ±0.04*	17.20 ±0.12*	3.19 ±0.08	2.02 ±0.02	48.44 ±0.03*	1.56 ±0.01	46.88 ±0.02*
WB	4.50 ±0.10*	16.56 ±0.09*	3.01 ±0.03	2.99 ±0.07	46.52 ±0.10*	1.40 ±0.02	45.12 ±0.06*

Note: IDF – insoluble dietary fiber, SDF, soluble dietary fiber, TDF total dietary fiber, * denotes statistically significant difference at p <0.05 level.

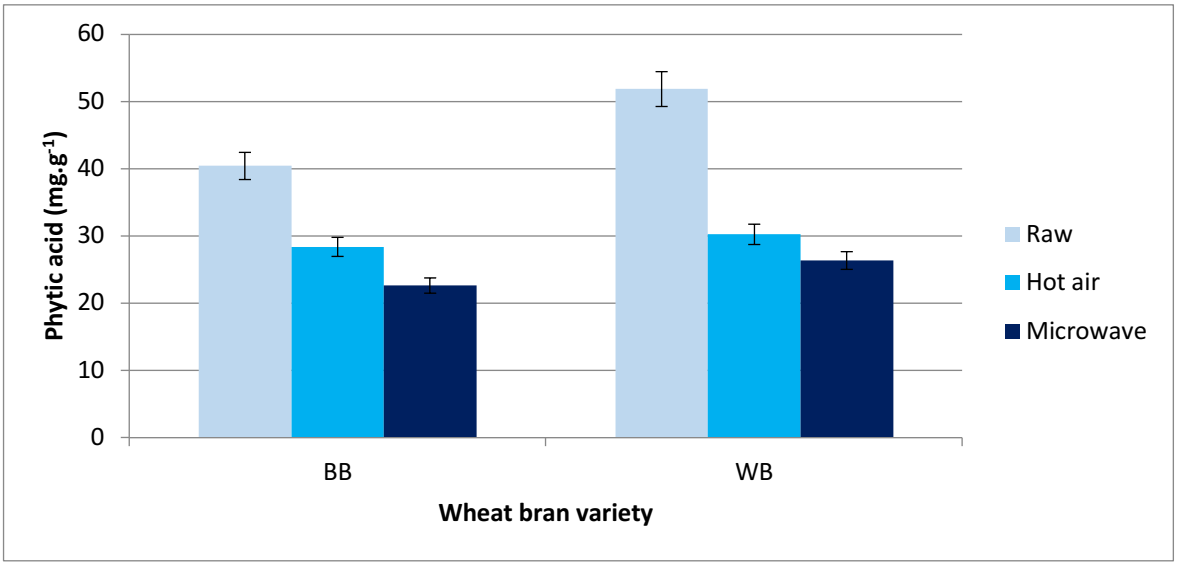

Figure 1 Phytic acid content in raw and treated wheat bran.

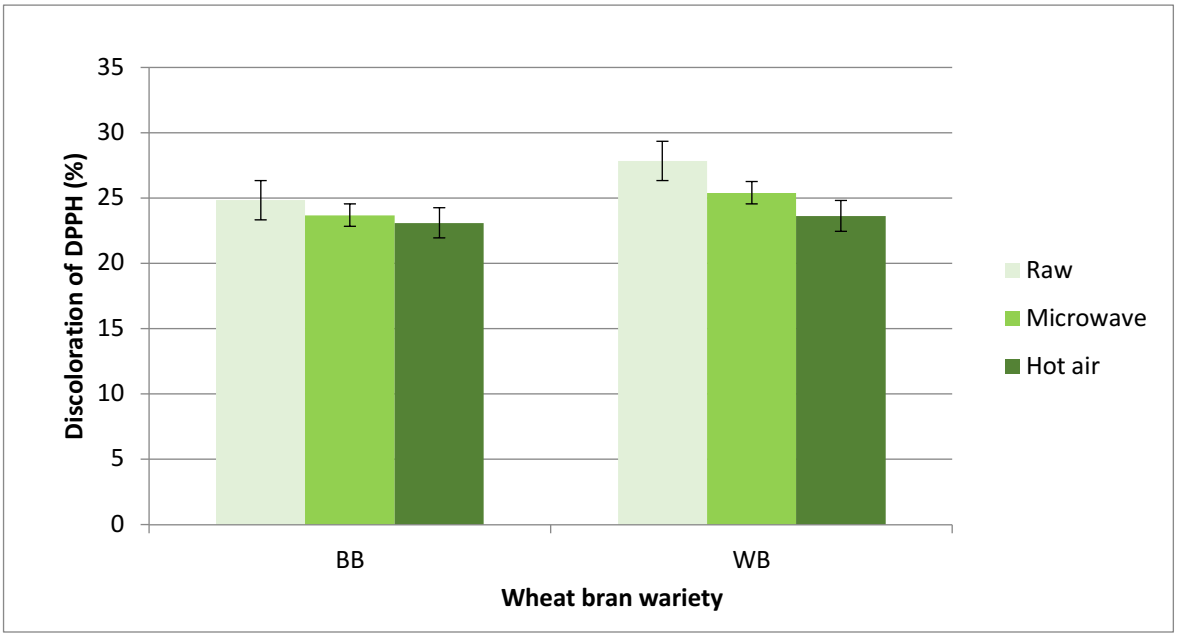

Figure 2 Discoloration of DPPH in raw and treated bran.

Table 2 Functional properties of wheat bran.

	WAC ($g.g^{-1}$)	WRC ($g.g^{-1}$)	SC ($cm^3.g^{-1}$)	OAC ($g.g^{-1}$)	LA-SRC (%)	SU-SRC (%)	Na-SRC (%)
Raw bran							
BB	2.46 ±0.03	1.74 ±0.01	4.21 ±0.03	1.49 ±0.02	141.98 ±4.21	183.50 ±1.65	182.41 ±3.15
WB	2.57 ±0.01	2.16 ±0.01	4.40 ±0.02	1.33 ±0.00	142.79 ±3.47	161.52 ±2.54	176.17 ±2.31
Hot air treated bran							
BB	2.99 ±0.00*	2.38 ±0.00*	5.22 ±0.06*	1.48 ±0.02	174.44 ±4.06*	199.74 ±3.02*	177.84 ±3.01*
WB	3.19 ±0.04*	2.63 ±0.01*	5.41 ±0.01*	1.39 ±0.01	198.11 ±1.86*	214.54 ±1.09*	163.54 ±2.65*
Microwave treated bran							
BB	2.54 ±0.01	1.97 ±0.00	5.66 ±0.03*	1.57 ±0.01	154.44 ±2.71*	195.70 ±2.57*	164.52 ±4.16*
WB	3.13 ±0.02*	2.34 ±0.01	5.75 ±0.02*	1.39 ±0.00	172.12 ±2.48*	224.56 ±3.63*	167.88 ±2.45*

Note: OAC – oil absorption capacity, SC – swelling capacity, LA-SRC – lactic acid retention capacity, Na-SRC – sodium carbonate retention capacity, SU-SRC – sucrose retention capacity, WAC – water absorption capacity, WRC – water retention capacity, * denotes statistically significant difference at $p < 0.05$ level.

Table 3 Color parameters of wheat bran.

	L	*a*	*b*	*C*	*H*
Raw bran					
BB	63.33 ±0.62	8.57 ±0.14	13.50 ±0.22	14.58 ±0.26	57.59 ±0.06
WB	65.84 ±0.02	8.41 ±0.06	14.53 ±0.07	16.44 ±0.09	59.25 ±0.22
Hot air treated bran					
BB	59.87 ±0.04*	8.61 ±0.05	12.62 ±0.07*	14.95 ±0.45	55.68 ±0.05*
WB	58.60 ±0.62*	9.25 ±0.10*	14.16 ±0.30*	17.17 ±0.22*	57.38 ±0.21*
Microwave treated bran					
BB	61.79 ±0.76*	8.63 ±0.19	13.24 ±0.31	15.74 ±0.36	57.19 ±0.14*
WB	64.39 ±0.70	8.90 ±0.05*	14.46 ±0.21	17.40 ±0.28*	59.23 ±0.51

Note: * denotes statistically significant difference at $p < 0.05$ level.

SC indicates how much the fiber matrix swells as water is absorbed, including loosely associated water. It is a consequence of the macromolecule relaxation during hydration, which leads to an increment in the occupied volume by the fiber product. The greater capacity to swell is the most desirable parameter for the physiological functionality of DF **(Lebesi and Tzia, 2012)**. The SC of raw bran was higher (4.21 and 4.40 $cm^3.g^{-1}$) than obtained **Mora et al. (2013)** (2.92 $cm^3.g^{-1}$) for wheat bran. From the results concluded that thermal treatment significantly increased the SC of wheat bran. The highest SC (5.66 and 5.75 $cm^3.g^{-1}$) was recorded after microwave treating of bran. The increase in SC might be attributed to a rise in the amount of short chains and the surface area of DF induced by thermal processing **(Dong et al., 2019)**.

OAC is the capability of dietary fiber to adsorb fat. During food processing, the reduction of cholesterol level in blood is linked with higher value of OAC **(Khan et al., 2018)**. Oil absorption of cereal derivatives, e.g., wheat bran, is related mainly to the surface properties of the bran particles but may also be related to the overall charge density and to the hydrophilic nature of the constituents **(Elleuch et al., 2011)**. It was observed that OAC of raw BB bran was higher (1.33 $g.g^{-1}$) than WB bran (1.49 $g.g^{-1}$). These values were lower compared to values observed by **Mora et al. (2013)** for wheat bran (2.18 $g.g^{-1}$). Authors **Ma and Mu (2016)** describe that low OAC values might be due to the absence or limited presence of lignin. The results showed that treatment of bran using both methods had no significant effect on OAC values.

The SRC method has been conceived to produce a combined pattern of the four SRC values to establish a practical flour quality/functionality profile. It is clear that fibre functionality in food formulations derived from its interaction and spatial arrangement within the biopolymers system **(Rosell, Santos and Collar, 2009)**. Wheat bran is increasingly added to mostly cereal-based food products (bread, cookies, breakfast cereals, pasta, snacks, cakes, and more) **(Hemdane et al., 2016)**. For this reason the selected SRC of wheat bran samples and the effect of bran treatment were also evaluated. The SRC values are summarized in Table 2. Generally, lactic acid SRC is associated with glutenin characteristics, sodium carbonate SRC is related to levels of damaged starch, and sucrose SRC with pentosan characteristics **(Rosell, Santos and Collar, 2009)**. The results showed that thermal treated bran had significantly higher lactic acid and sucrose SRC compared to raw bran. The highest values of lactic acid SRC were observed after hot air heating (174.44 and 198.11% for BB and WB, respectively). Furthermore, the

results revealed that microwave treated WB bran had the highest sucrose SRC (224.56%). On the other hand, the sodium carbonate SRC decreased after thermal treatment process up to 163.25% (hot air heated WB).

Color parameters

Color is an important visual quality (attribute) of food products **(Ferreira, Chang and Steel, 2011)**. The color attributes of raw and treated bran are summarized in Table 3. The lightness values of raw bran (63.33 and 65.84 for BB and WB, respectively) were similar to results described by **Onipe, Beswa and Jideani (2017)**. Results showed that thermal treatment of bran samples decreased the lightness value of bran. Moreover, the lowest lightness values (58.60 and 59.87 for WB and BB, respectively) were recorded after hot air heating. It was also observed that after treatment of bran using both methods the redness of bran increased, while yellowness of bran decreased. Chroma (*C*), considered the quantitative attribute of colorfulness, is used to determine the degree of difference of a hue in comparison to a grey color with the same lightness **(Minarovičová et al., 2019)**. The results suggested that treated bran using both methods had higher *C* value compared to raw bran samples. The higher the *C* value, the higher is the color intensity of samples perceived by humans **(Minarovičová et al., 2019)**.

CONCLUSION

Wheat bran is the main source of dietary fiber in bakery products. The results showed that microwave and hot air heating altered the soluble and insoluble dietary fiber content. Hot air treatment significantly increased the total dietary fiber content up to 49.74%. The loss of phytic acid content was more than 44 % after microwave heating. The thermal treatment modified the functional properties of wheat bran, which are important in food processing. Wheat bran treated using hot air had higher water absorption and water retention capacity compared to wheat bran treated using microwave heating. Hot air heating significantly altered the color parameters of treated bran. From this study resulted that treatment of wheat bran using hot air heating significantly improved the wheat bran functional and physico-chemical properties.

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NEW TRENDS AND INNOVATIVE APPROACHES IN PERSONNEL MANAGEMENT OF FOOD BUSINESSES IN SLOVAKIA

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ABSTRACT
New practices and changes are appearing in personnel management in the same way as they occur in other areas of management. New trends, which have various ways of implementation, emerge, along with its impact on employees, managers and businesses. Employees are an important part of each business and therefore, it is important to have right people in right places. The objective of the paper is to find out how food businesses working in Slovakia are implementing an innovative approach to the personnel management. The research actively included 453 food businesses from all Slovakia. We decided to do research into these businesses because they are household names in the field of production of domestic food products. Production of high-quality domestic food products is considered important not only for people´s nutrition and availability of quality domestic products but also due to sustainability of the employment rate in this field, development and recruitment of professional, qualified and engaged employees. The statistical relations and correlations between variables were performed by Cronbach alpha, Spearman test, Kruskal-Walis test using programs EXCEL and SAS Enterprise Guide 7.1. We found out that food businesses in Slovakia had already started to implement innovative approaches to the personnel management, but there are still significant reserves and shortcomings. The positive aspect is that food business managers have understood importance of personnel management innovation because nowadays, their main task to find, recruit, select and retain a prospective employee.

Keywords: innovation; news trends; personnel management; food businesses

INTRODUCTION
Personnel management focuses on all the activities connected with a person in a working process. The most important functions are as follows: planning of human resources, recruitment and selection of employees, hiring and adaptation, development and education, evaluation and remuneration, communication and motivation of employees. The main goal of the human resource management is to procure employees that are necessary for occupying job vacancies within an organisation.

The global aim of the management of human resources is to achieve a competitive advantage through strategic distribution of capable and dedicated to the organization employees by using an integrated system of cultural personnel procedures **(Šajbidorová, Lušňáková and Dobišová, 2016)**. The first key to organisation´s success is *„to perceive the value and significance of employees and human resources and to understand that people represent the greatest asset of an organisation and its management decides whether an organisation will succeed or not"* **(Wroblovská, 2016)**. Human resources have become a core of each single business.

Current practice on human resource management refers to what human resource managers and line managers usually do **(Juríčková, Kapsdorferová and Kadlečíková, 2018)**. The attention is nowadays paid to the need for strategic and systematic human resource management application which creates an added value within an organisation through effective and efficient use of human resources. In this regard, various new approaches have emerged, for example, human capital management, enhancing loyalty, talent management, ability oriented management, electronic human resource management, creating systems focused on high performance, work performance management or performance-based remuneration. A modern approach to human resource management relates to deployment of employees within a company, where the primary working profile of workers, their work orientation and preferences are of the highest importance. The tasks to which an employee has dispositions and duties are adapted accordingly. This practice leads to optimum use of work ability and an employee manages to contribute to fulfilment of company goals while boosting work effectiveness **(Měrtlová, 2015)**.

Employee recruitment procedures aims to collect a sufficient number of adequate candidates for a job vacancy from whom, by adequate selection methods, an organisation selects the most appropriate candidate or candidates to occupy the work position. The basic decision is which segment of the labour market we focus on and what means of communication we select **(Frischmann and Žufan, 2017)**.

Recruitment of employees is an activity which ensures that job vacancies within a company attract a sufficient number of adequate applicants herefor, with adequate costs and in adequate time (on time) **(Koubek, 2015)**. During recruitment of employees, we might face "conflict of interests", of both, an employer, who needs to satisfy certain requirements and an applicant, who seeks new employment. On the other side, there might company employees who want to change their current work environment in the company. The study by **Diesel and Scheepers (2019)** gives human resource management insight into strategically directing leadership recruitment and development towards creating an organisational climate to enhance ambidexterity.

Present trends in employees´ recruitment developed back in 2004, when there was a significant technological shift in the area of the Internet communication and the era, called web development, started while hard website content was being replaced by shared content. **Blštáková et al. (2015)** wrote some methods, which actually show the highest usage potential, and as such has been proved to many companies:

- job recruiting websites of a company,
- professional webs,
- social sites,
- direct and indirect addressing of candidates.

Managers and leaders are provided with an opportunity to communicate across the market via social sites and therefore search for new employees. The biggest professional social site LinkedIn is mostly used by human resource managers when searching for professionals**.** Direct addressing of selected individuals is usually the last phase from the set of other methods, such as references of current employees, cooperation with educational institutions, etc. **(Koubek, 2015)**. Due to complexity of this method, new companies dealing with headhunting are slowly emerging and other businesses can hire such companies for searching for their prospective employees. The headhunting method can be demanding especially for leaders.

Employees´ selection can be placed between recruitment and hiring of employees. The objective of employees´ selection is to make the analysis and sort out applicants for a specific work position, compare them with requirements and demands of an organisation, and select the best possible applicant for a job vacancy.

The employees´ selection process is specific because a human resource manager has to sort out the best possible candidate from the given list of applications, the one who would best fulfil specific working criteria. At the same time, it entails personal as well as professional characteristics of applicants as well as their qualification potential and flexibility **(Mužík and Krpálek, 2017)**. An assessment centre belongs to the most frequently used methods within employees´ selection – it is a systematic selection tool, evaluating abilities of an applicant for a work position by comparing their knowledge, abilities, skills with requirements for their performance regarding a specific work position. This type of employees´ selection is mainly performed when occupying top management positions **(Vetráková and Bočincová, 2013)**. Nowadays, traditional work interviews have been moved to informal environment, e.g. lunch and it provides a unique view of candidate´s character. To make companies test the skills of candidates in a standardised way, they use for example 3D simulation of work environment in the virtual reality. New trends in employees´ selection and technology development has promoted creation of so-called video-recruitment.

In general, selection of employees via the Internet is seen more effective in contrast to offline methods. Considering the higher number of applicants, it provides the managers with the access to various applicants. It covers a shorter recruitment period and helps to cut overall recruitment costs **(Christiansen, et al., 2017)**. Talented people from abroad are often recruited via video-interviews.

Employees´ selection through information – communication technologies is likely to boost effectiveness and influence, cuts costs or increases the capacity in the selection process of the most appropriate candidate. In general, digital or online recruitment and the upcoming selection can help to reach a wider potential as well as promote the life cycle of selection **(Zeuch, 2016)**.

Within employees´ selection one cannot omit more and more active use of diagnostic methods, evaluating personal characteristics of applicants for work position and having a significant influence on performance and work effectiveness **(Evangelu, 2008)**.

Scientific hypothesis

In order to evaluate the level of active work in food businesses operating in Slovakia and implementation of new trends within personnel management, we have stated and, based on the analysis in question, verified the following research assumptions:

Searching and recruitment of employees:

A1: We assume, that companies deliberately pay attention to planning process of selection and employees´ recruitment.

A2: We assume, that companies, which deliberately plan the process of searching and recruiting of employees in companies, consequently prepare and carry out selection of employees.

Selection of employees:

A3: We assume that companies which deliberately prepare and carry out selection of employees, consequently accept principles of diversity of applicants within their selection.

A4: We assume, that companies, which deliberately prepare and carry out selection of employees, apply innovative methods of employees´ selection (assessment centre, chat bots, video interviews, diagnostic methods).

MATERIAL AND METHODOLOGY

Today´s concept of human resource management has started to evolve in order to form the core of the whole management. This new approach has helped to enhance significance of a human being as a key to organisation´s

success. Human resource management no longer contains only strategic aspects but focuses on external factors of formation and placement of people within an organisation as well, e.g. population development, labour market, value orientation and social conditions.

The aim of the paper is to assess implementation of these innovative approaches within specific areas of personnel management in food businesses across Slovakia.

Primary research was focused on activities within personnel management in food businesses working in Slovakia. Based on the data available on the website of the Statistical Office of the Slovak Republic, in the first quarter of 2019 there were 587 988 economic subjects in Slovakia. Out of which 4434 subjects were working, according to the statistical classification of economic activities SK NACE, in the sector of CA „Food, beverages and tobacco production. Even though the given number represents only 0.75% share of all the economic subjects, food businesses in Slovakia are an important part of national economy and they significantly influence production of domestic foodstuffs and beverages and at the same time they provide work positions for population.

A questionnaire called „New trends in human resource management in food businesses" was created based on elaborated theoretical results. The first part consisted of identification questions such as a company size, legal form, capital share of the company and the region of Slovakia in which the company operates. Each area contained several questions and thanks to specific statements we found out whether a specific activity within human resources is performed by the companies. We also searched how new trend in individual parts of business are implemented. Positive, rather positive, neutral, rather negative or negative attitude to question was expressed on a five-level Likert scale.

The questionnaire was, within the pilot research in February 2019, tested on a sample of 20 companies in the Nitra region. After little modification, the final research was done from March to April 2019, the questionnaire was provided by a phone call arrangement or e-mail arrangement to 470 food businesses operating in Slovakia. The respondents who answered the questions were mostly the managers from human resource departments. In case that the organisation did not have a specific department for human resources, the questionnaire was filled in by the manager or the owner, responsible for this area. By the beginning of May 2019 we received electronic or a printed version of 453 filled questionnaires ready for use, elaboration and evaluation.

The research included 70 big, 125 middle, 148 small and 110 micro companies. From the organisational-legal business form point of view we focused exclusively on companies which are incorporated (160) or limited (293) because there are various businesses with different legal forms in Slovakia to control assumptions we wanted to ensure representativeness of a selection file as for the organisational-legal business forms.The research included 379 exclusively Slovak businesses, 35 exclusively foreign businesses and 39 businesses with a combined capital share. As for the territorial point of view, there were food businesses from all the eight regions of Slovakia.

The questionnaire´s answers were elaborated and classified which enabled consequent verification of the given research assumptions by selected mathematical-statistical methods and formulation of suggestions and recommendation for the practice.

Except for the questionnaire, we carried out managed structured interviews in selected food businesses from February to March, providing us with entrance to the issue of human resources and enabled us with quality analysis of the given issue from another point of view, even though the answers of the respondents can be considered as partially subjective.

Nevertheless, we used the method of monitoring, findings of which served as a contribution within discussion, formulation of suggestions and conclusions.

Statistical analysis

Processing the data, obtained by the questionnaire, was done through table processor MS EXCEL 2016. The data was evaluated through statistical software SAS Enterprise Guide 7.1.

The consistency of a selection file was found by Cronbach alpha coefficient. Cronbach alpha coefficient belongs to widely used methods of scale reliability evaluation and represents level of the internal consistency. If Cronbach alpha coefficient reaches values of 0.7 and more, it represents sufficient internal scale consistency **(Benda-Prokeinová, 2014)**.

From the data obtained from the questionnaire, where respondents had a possibility to express the level of approval or disapproval with the statement on a five-level Likert scale, we found the characteristics of a position. According to **Prokeinová (2010)**, through application of basic descriptive characteristics we gain values of modus and averages of individual respondents´ preferences.

In the next part of the research we applied correlation analysis. Correlation analysis represents, according to **Benda-Prokeinová (2014)**, statistical approach which describes a relationship between numeral variables. The more the absolute value of correlation coefficient reaches one, the stronger the dependence and vice versa, the more it reaches zero, the weaker the dependence.

For testing statistical hypothesis resulting from research assumptions, mathematical-statistical methods – non parametrical test were applied: Kruskal-Wallis test and Spearman coefficient. We searched for differences in answers of the questionnaire based on specific identification symbols, dependence between two variables, as well as the power of this dependence **(Markechová, Stehlíková and Tirpáková, 2011; Rimarčík, 2007)**.

Except for the questionnaire, we realized managed structured interviews in selected food businesses from February to March, providing us with the entrance to the issue of human resources and enabled us with quality analysis of the given issue from a different point of view, even though the answers of the respondents can be considered as partially subjective. Nevertheless, we used the method of observation, findings of which were a contribution for a discussion, formulation of suggestions and recommendations and making conclusions.

RESULTS AND DISCUSSION

Employees are an important part of each company, therefore it is important to have right people in right

places. To have a reliable and responsible employee is not easy, as labour market is currently facing a lack of qualified personnel and is also recording a limited amount of work offers. Nowadays, companies have to attract a potential employee, not vice versa. People have the opportunity to select from various work offers, they are interested in what an employer can offer them. An organisation needs to have a good reputation, a right strategy, organisation culture and quality system of motivation and remuneration of employees.

Searching and recruitment of employees

To find and obtain the most appropriate candidate to occupy work place is not an easy task. Present-day labour market is marked with globalization, ongoing technological progress and occupational competitiveness results in talent fight. Potential of an employee, thus level of their abilities and skills, becomes inevitable part of every society´s prosperity.

Situation on the labour market is, from the employers´ point of view, demanding, as a number of free work positions still surpasses number of unemployed. To hire a new employee is becoming more and more difficult and costly. Therefore, many companies start to realize, that it is more effective to keep those working in the companies.

Searching and recruiting best candidates for a position is an activity, which contains identification and searching adequate work sources, informing about vacant work positions in an organisation, providing these vacant work positions, in negotiation with candidates, in receiving adequate information about candidates and in organisation and administrative arrangement of all these activities.

In the 1st round of questions of the questionnaire, respondents expressed the level of their approval or disapproval on a five scale range within three statements regarding searching and recruitment of potential employees:

-Process of searching and recruitment of employees in company is planned and we pay target attention to it.
-Within searching and recruitment of employees we use even innovative forms and ways.
-Within searching and recruitment of employees we apply even active searching of „talents“ or „headhunting“(„hunting for brains“).

In two questions, the respondents selected more alternatives and could select one or more answers:

Searching and recruitment of employees is realized traditionally by:

-personnel agencies,
-cooperation with educational institutions,
-recommendations of employees,
-advertisement in media,
-Profesia.sk,
-other.

Searching and recruitment of employees is realized by new forms:

-Facebook,
-LinkedIn,
-Twitter,
-Instagram,
-others.

For more comprehensive interpretation of research results we selected characteristics of average and modus, which can be used within questions with the possibility of answers on a five scale range. In the first round there were three such questions and their average and most common answers are showed in the Figure 1.

The bar graph represents average answers of respondents (data is shown in the basis of the graph) and the line graph shows the most common answers of respondents (data is shown above the graph).

Statement „Process of searching and recruitment of employees in a company is planned and we pay a target attention to it“ is approved by the most of the respondents because 67.77% respondents answered either partially or absolutely agree with this statement. Only 4.19% of companies expressed that they absolutely disagree with the statement. An average answer was 3.98 regarding the answer „I partially agree“ and the most selected value was 5, representing absolute approval.

An average answer of the representatives of food businesses within the statement „Within searching and recruitment of employees we use even innovative forms and ways“ reached 2.97, representing answer I rather agree than disagree and at the same time modus represented the same value. From the overall number of companies only 15.23% absolutely agreed with this statement and 18.10% absolutely disagreed with the statement.

The statement „Within searching and recruitment of employees we apply even active searching for talents or headhunting (hunting for managers)“ reached absolute disapproval of 43.7% companies. Absolute approval was expressed by 9.27% and partial approval was expressed by 8.6% companies. An average value selected was 2.2, representing possibility „I partially disagree“ and the most frequently selected value was „I absolutely disagree“ (1).

Except for the questions where respondents had a possibility to express level of approval or disapproval with the statement, there were, within the first round, „searching and recruitment of employees“ included two questions with more possibilities of answers, from which the respondents could select one or more answers. As the results of the research show, 53.8% companies within searching and recruitment of employees by traditional methods, they rely on recommendations of their employees. Some more than 40% of companies use advertising in media and portal www.profesia.sk. Personnel agencies are used by 20.5% of companies and using Office of Labour, Social Affairs and Family is used by 5% of companies for searching for employees.

If companies implement even innovative ways and possibilities of searching and recruitment of employees, 67.4% of them use social network of Facebook, 13.6% Instagram and 9.8% LinkedIn. Almost 20% of the representatives stated that their company does not use any innovative forms and ways of searching and recruitment of employees.

In the next step, using SAS Enterprise Guide 7.1, we realized Kruskal-Wallis test, to find out whether there are statistically significant differences among the answers of companies according to their size, legal form of business, capital participation of business or region, in which the company operates.

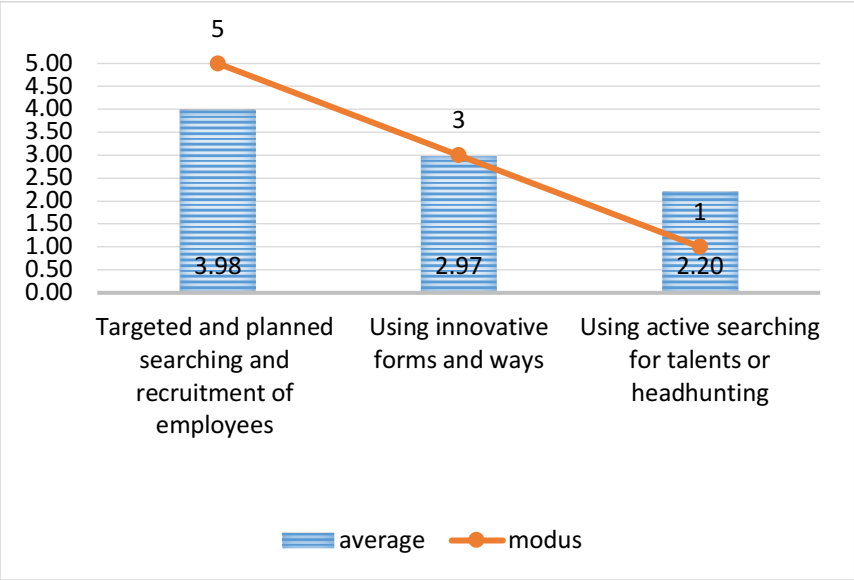

Figure 1 Characteristics of postion evaluating searching and recruitment of employees.

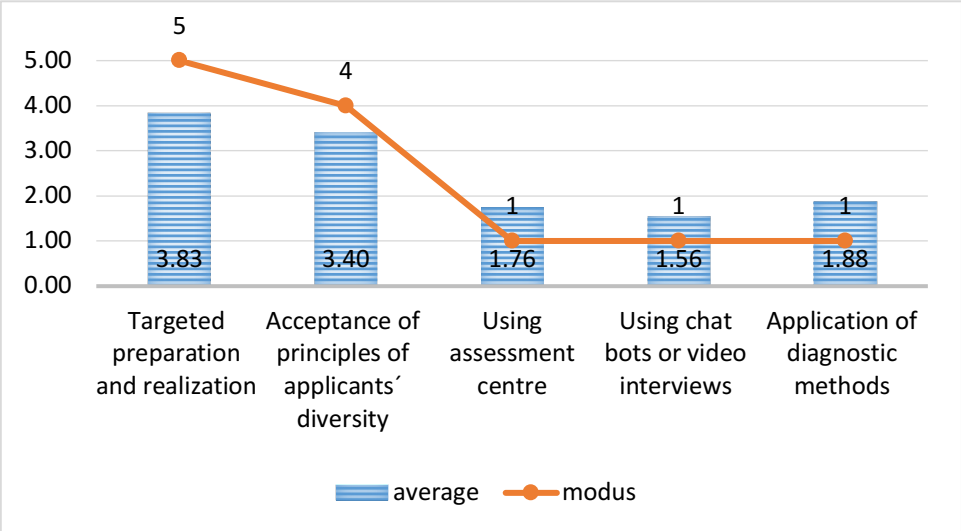

Figure 2 Characteristics of the position evaluating employees´ selection.

Table 1 Results of Kruskal-Wallis test – searching and recruitment of employees.

	Values of Kruskal-Wallis test according to			
	Company size	**Legal form of business**	**Capital participation of the company**	**Region**
Targeted and planned searching and recruitment of employees	<0.0001**	0.6876	<0.0001**	0.1637
Using innovative forms and ways	0.0025**	0.1494	<0.0001**	0.2089
Using active searching talents or headhunting	<0.0001**	0.0562	<0.0001**	0.0494*

Note: * statistically significant differences on the level of significance 0.05. ** highly statistically significant differences on the level of significance 0.01.

The results of Kruskal-Wallis test are shown in the Table 1. Values marked as „*“ represent statistically significant differences on the significance level 0.05 and values marked as „**“ represent highly statistically significant differences on a significance level 0.01.

In case of targeted and planned searching and recruitment of employees, we can see that within the company size and capital participation of the company, the value of Kruskal-Wallis test was lower than 0.0001. Thus, there are statistically significant differences in the answers pf companies based on their size as well as capital participation of companies. Legal form of business and region, in which the company operates, does not have an influence on targeted and planned searching and recruitment of employees and vice versa – size of a company and capital participation does have an influence on it.

Based on Kruskal-Wallis test, we can state that size of a company and its capital participation has influence on using innovative forms and ways of searching and recruitment of employees. We can state that legal form of business sand region in which the company operates, do not influence using innovative forms and ways within searching and recruitment of employees.

Application of active searching of talents or headhunting within searching and recruitment of employees is not influenced only by legal form of business. Thus, there is no difference in whether the company is incorporated or limited, from the statistical point of view there are no differences in answers of these companies. However, in case of the region, in which the company operates, there are statistically significant differences on a significance level of 0.05. We can state, that size of company, capital participation and the region in which the company operates have influence on application of active searching of talents or headhunting.

To find out the power of dependence among individual questions from the questionnaire of the research, we used SAS Enterprise Guide 7.1 values of Spearman correlation coefficient for combination of questions. Values marked with “**” mean, that they are highly statistically significant at the level of significance 0.01.

As the first, we found out power of dependence among the questions of the first round. The values can be seen in Table 2. The highest correlation reaches value of 0.36598 and consequently is marked as „**“ meaning that it is highly statistically significant. Based on the interpretation of correlation coefficients according to **De Vaus (2002)**, this statistical dependence between the third and the fifth question, is middle to significant. We can state, that there is middle to significant positive dependence between using innovative forms and ways of searching and recruitment of employees and application of active searching of talents or headhunting. The remaining two correlations are highly statistically significant as well but represent low or middle dependence.

Except dependences among questions of the first round we were interested in dependences among questions of the first and the second round. Values of Spearman correlation coefficient are given in the Table 3. Values marked with “**” mean, that they are highly statistically significant at the level of significance Alpha = 0.01. Values marked with “*” mean, that they are statistically significant at the level of significance Alpha = 0.05. The highest dependence can be seen between the questions 1 and 6, where the value found, was 0.47605 which is highly statistically significant and represents middle to significant dependence. We can state, that between planned searching and recruitment of employees and targeted preparation and detailed realization of selection of employees, there is middle to significant dependence.

Selection of employees

The main objective of the employees´ selection is to perform analysis and sort out applicants for specific position, compare them with the requirements and requests of the organisation, and to select the applicant who would be the best choice for the organisation.

Within the employees´ selection we can use an approach characteristic for modern human resource management, consisting of the fact that it pays more attention to whether the applicants fulfil conditions of specific position as well as requirements of organisation as a whole. These requirements include even ability to work more effectively as a member of a team. These characteristics are needed to be taken into account in case that an applicant is a member of the TOP management of the organisation, or if their position is in production.

Within the second round of questions of the questionnaire, regarding employees´ selection, and application of new trends within selection, the respondents expressed level of approval or disapproval with the following statements:

-Employees´ selection is prepared and realized in a detail.
-Within employees´ selection we accept principles of applicants´ diversity.
-Employees´ selection is done by assessment centre.
-Within employees´ selection we use chat bots or video interviews.
-Within employees´ selection we apply even diagnostic methods (such as personal cards packet, table of requested work, motivation table, creative pattern, brain teasers, etc.).

Average answers and the most common answers of companies´ representatives are shown in the Figure 2. The bar graph represents average answers of respondents (data is given in the basis of the graph) and the line graph shows the most common answers of the respondents (data is given above the graph).

The most common value selected, expressing respondent´s answer to the statement „Employees´ selection is prepared and realized in a detail“ was value „5“ „I absolutely agree“. This value was selected by 37.5% of managers. The value „4“ „I partially agree“ was selected by 28.03% companies, representing together 65.53%. Average selected value was 3.83. It represents answer „I partially agree“. Only 5.73% of the respondents absolutely disapproved of this statement. Average value of approval with the statement „Within employees´ selection we accept principles of applicants´ diversity“ was 3.40. This one is between the answers 3 and 4, closer to 3 expressing the answer „I rather agree than disagree“. The most common answer reached the value of 4, so 30.63% companies „partially agree“ with the statement. Absolute approval was expressed by 19.20% companies. Together,

the value represents 49.83% of companies, which partially or absolutely agreed with the statement.

Statement „Employees´ selection is done by assessment centre“ was absolutely approved by only 19.20%. Up to 58.28% of the respondents absolutely disapproved of this statement, representing the most common answers at the same time. Partial agreement was expressed by 20.75% of the respondents, representing together 79.03% of the companies, which partially or absolutely do not use assessment centre within employees´ selection. Average answer had a value of 1.76 – closest to the answer „I partially disagree“.

The average value selected within statement „Within employees selection we use chat bots or video interviews“ was value of 1.56. It ranks among the answers „I absolutely disagree“ „I partially disagree“ (values 1 and 2). The most common answer was „I absolutely disagree“, that was the answer of 73.51% of the respondents. 3.09% of the respondents absolutely agreed and 5.96% agreed partially. Together, only 6.05% agreed partially or absolutely.

The most common answer to the statement „Within employees´ selection we apply even diagnostic methods (such as personal cards packet, table of requested work, motivation table, creative pattern, brain teasers, etc.“ was, same like within the previous two answers, „I absolutely disagree“, stated by 55.85% of the representatives of companies. Average answer was 1.88 of the value. 18.32% of the companies expressed that the „partially disagree“. Only 4.41% of the companies absolutely approved and 8.83% partially agreed.

With the aim to find out, whether there are statistically significant differences between the answers of the respondents of the companies according to the size, legal form of business, capital participation of the company or region, in which the company operates, we performed, same as before, Kruskal-Wallis test. The results of the test are shown in Table 4. The values marked as „*“ represent statistically significant differences on the significance level 0.05 and values marked as „**“ represent highly statistically significant differences on the significance level of $\alpha = 0.01$.

Based on the realised Kruskal-Wallis test, we can state that size of company and capital participation have significant influence on targeted preparation and detailed realization of employees´ selection, as their value is lower than 0.0001 and we can confirm highly statistically significant differences. The region, where the company operates has influence on targeted preparation and detailed realization of employees´ selection. The value 0.0107 represents statistically significant differences in the answers of respondents. Legal form of business in this case does not have statistically significant influence.

In case of acceptance of diversity principles, from table it is clear that within size of company, legal form of business as well as capital participation, the value is lower than 0.0001, which proves the existence of highly statistically significant differences. As for the region, in which the company operates, the value is 0.0327, representing statistically significant differences. Based on our findings we can state that, all four factors have influence on acceptance of applicants´ diversity principles within selection of employees.

Table 4 shows that using assessment centre within employees´ selection is influenced by all the factors (size of company, legal form of business, capital participation of company and region in which the company operates), because within all four the result of Kruskal-Wallis test is smaller than 0.01. There are highly statistically significant differences in answers of companies on significance level 0.01.

In case of using chat bots or video interviews there are highly statistically significant differences within size of company and capital participation of company. We can state that legal form of business and region where the company operates, do not have influence, and on the contrary, size of company and capital participation of company have a significant influence on using chat bots or video interviews within employees´ selection.

Application of diagnostic methods within employees´ selection is significantly influenced by one identification symbol. Within all the factors there is a value lower than 0.01. Thus, in every case, there are highly statistically significant differences in the answers of the respondents.

Within the topic of „employees´ selection“, identification symbols of companies show significant influence on respondents´ answers.

Another research continued by finding values of Spearman correlation coefficient in order to find out the power of dependence among the questions of the second round.

Relationship among questions of the first and the second round was already solved and it is given above within the issue of „Searching and recruitment of employees“.

Values, showed by relationship among questions of the second round, are in Table 5. Values marked with “**” mean, that they are highly statistically significant at the level of significance 0.01. The highest value in the table expressing dependence, has a value of 0.52563 and at the same time it is highly statistically significant. It represents significant to very strong dependence between using assessment centre and at the same time using chat bots or video interviews within selection of employees.

The second highest correlation has a value of 0.44927, it is highly statistically significant and it ranks among questions 9 and 10. It represents middle to significant dependence between using chat bots or video interviews and applying diagnostic methods within employees´ selection. In the table there are other two correlations which are from then category of middle to significant, they are grey colour and they both are highly statistically significant. From the rest of the correlations there are four highly statistically significant, they represent low to middle dependence and two of them are not statistically significant. All the correlations in Table 5 belong to positive correlations.

Verification of the given research assumptions

Within the sub-chapter „Material and methods“ we formulated research assumptions. Based on realized analysis and mathematical – statistical methods they were verified and the results are as follows:

Table 2 Values of Spearman correlation coefficient for questions of the firs round.

	Question 1	Question 3	Question 5
Question 1	1.00000	0.29455**	0.19779**
Question 3	0.29455**	1.00000	0.36598**
Question 5	0.19779**	0.36598**	1.00000

Note: ** highly statistically significant on the level of significance 0.01.

Table 3 Values of Spearman correlation coefficient for questions from the first and the second round.

	Question 6	Question 7	Question 8	Question 9	Question 10
Question 1	**0.47605****	0.26992**	0.10504*	0.00987	0.13867**
Question 3	0.25829**	0.23726**	0.29350**	0.23360**	0.22435**
Question 5	0.18453**	0.21970**	0.44102**	0.40921**	0.41106**

Note: * statistically significant on the level of significance 0.05. ** highly statistically significant on the level of significance 0.01.

Table 4 Results of Kruskal-Wallis test – employees´selection.

	Values of Kruskal-Wallis test according to			
	Size of company	Legal form of business	Capital participation of company	Region
Preparation and detailed realization	<0.0001**	0.4431	<0.0001**	0.0107*
Acceptance of principles of applicants´ diversity	<0.0001**	<0.0001**	<0.0001**	0.0327*
Using assessment centre	<0.0001**	0.0023**	<0.0001**	<0.0001**
Using chat bots or video interviews	0.0051**	0.1798	0.0004**	0.2183
Application of diagnostic methods	<0.0001**	0.0040**	<0.0001**	0.0008**

Note: * statistically significant differences on the level of significance 0.05. ** highly statistically significant differences on the level of significance 0.01.

Table 5 Values of Spearman correlation coefficient for questions of the second round.

	Question 6	Question 7	Question 8	Question 9	Question 10
Question 6	1.00000	0.37320**	0.18601**	0.06095	0.16014**
Question 7	0.37320**	1.00000	0.15239**	0.05211	0.16284**
Question 8	0.18601**	0.15239**	1.00000	**0.52563****	0.41664**
Question 9	0.06095	0.05211	**0.52563****	1.00000	0.44927**
Question 10	0.16014**	0.16284**	0.41664**	0.44927**	1.00000

Note: ** highly statistically significant at the level of significance 0.01.

Searching and recruitment of employees

A1: We assume that companies deliberately pay attention to planning process of searching and recruitment of employees.

The first research assumption was accepted. Based on characteristics of the position of answers we state that two thirds of respondents partially or absolutely agreed with the statement about planning process of searching and recruitment of employees of company.

A2: We assume that companies which plan the process of searching and recruitment of employees in company, plan and realize selection of employees as well.

The second significant assumption was accepted based on values of Spearman correlation coefficient which proved positive dependence between the target planned process of searching and recruitment of employees and of detailed preparation and realization of employees selection, of providing possibilities of their improvement, of providing possibilities for their development and education and at the same time perceiving communication as an important attribute of cooperation in the company and outside. The strongest dependence among the three given above was proved between planning process of searching and recruitment of employees and detailed preparation and realization of employees´ selection.

Employees´ selection

A3: We assume that companies, which deliberately prepare and realize employees´ selection, at the same time accept applicants´ diversity principles within their selection.

The third research assumption was accepted. If companies deliberately prepare and realize process of employees´ selection, they try to accept and follow applicants´ diversity principles.

A4: We assume that companies that deliberately prepare and realize employees´ selection, apply innovative methods of employees´ selection (assessment centre, chat bots, video interviews, diagnostic methods).

The fourth research assumption was not accepted. We suppose that those companies which prepare and realize employees´ selection, will, within this kind of activity use innovative methods. We found out only very low dependence between preparation and realization of employees´ selection by using assessment centre as well as applying diagnostic methods. In case of using chat bots and video interviews there was no statistically significant influence found. Based on literature sources we assumed that assessment centre is very used method within process of employees´ selection. Based on our findings we can therefore state that between preparation and realization of employees´ selection and using assessment centre there is above mentioned positive dependence.

The process of economic growth greatly depends on the qualification and use of human resources, of the creative, dynamic capacity of the human factor in the unfolding of economic life **(Lušňáková et al, 2018)**. According to **Armstrong and Taylor (2015)**, intention of human resources is to ensure that organisation has employees who are necessary to fulfil the entrepreneurial goals. Therefore, the objective is to ensure competitive advantages of organisation through recruitment, stabilization and development of employees. The diversity of jobs and lack of candidates force the employers to find creative ways to recruit new employees **(Briscariu, 2019)**.

Among the human resources processes, recruitment has been considered to be one of the most important ones. Many theories nowadays emphasize the importance of recruitment practices in the welfare of an organisation, stressing that its impact is crucial even in the business financial performance **(Vejsiu, 2019)**. Process of employees´ recruitment should start with planning, establishing number and time span of the necessity to occupy vacant positions. Then it leads to time span of addressing applicants from the external and internal labour market. The advantage of internal searching is the acquaintance of employees, support for flexibility and internal mobility reflected in career development, stabilization, and improvement of work ethics **(Lawrel and Boudreau, 2009)**. As for our research, food businesses in Slovakia which were included into research of using new trends in human resource management, they expressed their effort to plan human resources in company, however some of the addressed representatives of companies did not know exactly how to define what human resource management includes. Especially representatives of domestic micro and small companies (mostly owners) explained that because of exceeded bureaucracy and duties, they have no time left for systematic work with human resources.

In spite of the fact that the analysed food businesses in Slovakia plan the process of searching and recruitment of employees and they pay attention to it, the representatives´ standpoint was neutral as for using innovative forms and ways of searching and recruitment of employees. The new ways include e-recruitment, which utilizes available computer network services, especially web sites and e-mail **(Šikýř, 2014)**. The Internet offers employers additional options for communicating with potential job seekers, such as creating and developing social networks (Facebook, LinkedIn, Myspace, and Google+), and virtual communities of users with common interests. Social networks enable job seekers to publish their professional and personal profiles and give employers the opportunity to reach out to suitable job candidates in their enterprise **(Vetráková et al., 2018)**. Companies within searching and recruitment of employees very occasionally apply active searching for talents or headhunting. This condition should therefore be changed. The reason is given by **Grenčíková (2015)** and she writes that quality work force is becoming a competitive advantage. It is an advantage especially for employers who offer interesting work conditions. The author did not mean financial evaluation but relationship between the employer and employees. According to **Lenčéšová et al. (2018)** it is complicated for the companies in Slovakia to find and obtain talented employees or professionals. This issue is not only connected with small and medium sized companies.

Social media serves as a mediator for the effect of external knowledge flows on firm innovativeness when firms attach high importance to modern HRM practices. Taken together, the findings of **de Zubielqui, Fryges and Jones (2019)** underscore the importance of modem HRM practices to enable knowledge inflows via social media to influence innovativeness.

Food businesses in Slovakia, according to our findings, prepare and realize employees´ selection. They, anyway, had neutral standpoint to acceptance of applicants´ diversity principles within their selection. **Horváthová, Bláha and Čopíková (2016)** on the other hand, write about necessity to manage cultural work forces and take into account individual and group differences in needs, in work styles and various aspirations. It is important to take steps for everybody to be satisfied, to provide ethic approach of all the employees, which is based on equality principle and we all have the same rights and deal equally with everyone regardless the sexual orientation, religion, race, political ideals and various beliefs.

The area of employees´ selection, according **Bělohlávek (2017)**, includes the main methods of interview, assessment centre and various types of tests on working ability. The selected companies within employees´ selection only occasionally use assessment centre, chat bots, video interviews and other innovative methods. **Lisá (2019)** states, that ability to predict, is very limited, during testing traditional curriculum vitae. Well-designed assessment centre best predicts success of an applicant on a work position but it can only be used within the most complicated work positions or with university based applicants.

Recruiting and selection process are the most interesting functions for western researchers when we write about green human resources management **(Shahriari and Hassanpoor, 2019)**. In commitment-based human resource management, employees are hired based on their knowledge, their expertise is developed, and they are empowered to take reasonable risks in the interest of long-term outcomes. HRM policies, which mediated by innovative work practices, enable firms to realize their strategic intention to engage in innovation **(Ko and Ma, 2019)**. Effective leadership of human capital is a major managerial issue. Hiring and keeping employees is key to sustainable competitive advantage **(Smith and Rupp, 2004)**.

CONCLUSION

The important assumption for working and prospering organisation is to ensure the whole process, from human resource planning through searching and recruitment of work force, employees´ selection, their adaptation, education and development, communication and motivation, support of creativity and work environment and all the other areas should be secured on the highest level possible within active implementation of innovative processes to all the areas of human resource management. Based on realized analysis we can see the effort of companies to understand importance of implementation of innovative processes within personnel management, however practical application of processes in practice is very rare. If company wants to work effectively, investment into planning, recruitment, development and sustainability of employees is the cleverest decision possible.

Resulting from the results of the analysis of implementation of innovative processes within personnel management in food businesses in Slovakia, we recommend to:

- provide professional planning of human resource management in short time and in ling time perspective regardless the size of company, organisation. legal form, capital participation and establishment,
- immediately start intensive using available, effective and especially financially less demanding innovative ways of searching and recruitment of employees, like for example social networks,
- think and implement strategy of obtaining the best employees, who would be willing to work for a company and remain there; for example by headhunting, together with searching for talents, necessary for the company to have,
- intensively search for information and opportunities to discuss things with professionals, educational institutions and other organisations, how to apply new processes to every kind of activity within work with human resources, as new trends are used by food businesses in each area of human resource management minimally or not at all,
- within employees´ selection, apply maximally principle of diversity and get rid of any prejudices, support employees´ selection through time less demanding and effective video interviews, role playing and with maximum usage of assessment centre and so forth.

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SUBSTANTIATION OF BASIC STAGES OF GLUTEN-FREE STEAMED BREAD PRODUCTION AND ITS INFLUENCE ON QUALITY OF FINISHED PRODUCT

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ABSTRACT

Development and introduction of high quality gluten-free products is one of the priorities of food industry. Feasibility of producing gluten-free steamed bread based on rice and corn flour using flaxseed, sunflower, sorghum and quinoa flour additives is proved in the article. Recommended ratios of flours are established: Frc:Ffs 95:5, Frc:Fsn 95:5, Fcn:Fqn 85:15, Fcn:Fsg 90:10. The parameters of dough kneading are studied and the influence of additives on relative elasticity, plasticity and resilience is established. Use of additives leads to a decrease in irreversible relative deformation of dough for 36 – 68% and relative plasticity for 16 – 18%, to increase of its elasticity relative resilience up to 2.3 times. Dough fermentation process is investigated. It is established that amount of carbon dioxide accumulated in gluten-free dough increases by 10 – 30%. Process of acid accumulation during fermentation is studied. A flow chart for the production of gluten-free steamed bread is proposed. The parameters for the production of gluten-free steamed bread were established and justified. Product is prepared in a single-phase method, adopted in practice of baking bread. The duration of dough mixing is 10 – 15 min, fermentation 20 – 35 min. Steam treatment is carried out under atmospheric pressure. Recommended steam processing time is 35 min for bread based on rice flour, 30 min for based on corn flour. In comparison with the traditional technological scheme, it is recommended to use a double boiler instead of an oven.

Keywords: gluten-free; steamed bread; rheology; dough slackness; fermentation

INTRODUCTION

The imbalance of the diet of the world population in modern economic conditions leads to a constant shortage of essential nutrients that are necessary for consumption, especially in case of diseases associated with gastrointestinal tract functioning. Unfortunately, today quality, biological value, safety and pricing of food products do not always meet the requirements of sick people. Therefore, the development and introduction of high quality gluten-free products is one of the priorities of food industry.

Production of specialized food products, free from certain ingredients, and gluten-free products are one of the largest segments of this market today. Use of structure-forming additives of polysaccharide nature in technologies of bakery, confectionery, macaroni products can simulate a structure similar to traditional flour products. The main drawback of such products is their reduced nutritional and biological value, lack of protein, vitamins and minerals. This problem can be solved by introduction of high protein ingredients, primarily soy isolates and concentrates, peanut protein isolates, lupine, caseinates, synthetic vitamins and trace elements, etc.

At present scientists have developed a wide range of gluten-free foods – breads, muffins, biscuits made of buckwheat and rice flour **(Havrylova, 2007; Drobot, Mykhonic and Hryschenko, 2010; Kuznetsova, 2010; Lazorenko, 2013; Dorokhovych, Lazorenko and Omelyanchenko, 2014)**. However, the segment of Ukrainian market of steamed bread production is still not developed. The fact is that steamed bread is a traditional Chinese bread made from wheat flour or its mixture with rice. This product is widely distributed in the eastern countries (mainly in China, Japan, Korea, Thailand), as well as in the USA, Canada and some European countries **(Huang et al., 1996)**. Its advantages include slowing of glucose absorption processes during steam treatment, which reduces glycemic index; absence of melanoid formation reaction, which reduces losses of lysine and other water-soluble amino acids; absence of harmful acrylamides that are not formed during steam treatment **(Addo et al., 1991; Liu et al., 2012; Zhang et al., 2014; Huang and Miskelly, 2016; Fu, Chang and Shiau, 2015)**.

To create a competitive technology of steamed bread scientists are currently searching for the following directions:

- adaptation and correction of world experience in the production of steamed bread;
- expansion of raw material base and assortment of gluten-free steamed bread products;
- improvement of structural and mechanical properties of steamed bread.

Understanding the main aspects of regulating the nutritional value of gluten-free steamed bread, forming its quality, regulating the properties and expediency of using enriching flour raw materials will contribute to developing products with high nutritional and organoleptic quality indicators available to all population groups.

There are three main types of traditional steam bread in China: Northern Type, Southern Type and Taiwan Type. Northern steamed bread is used as the main food product, while Southern and Taiwan, with a sweet taste, are used most as desserts **(Lin, Miskelly and Moss, 1990; Wang et al., 1995; Zhu et al., 1997; Fan et al., 2009; Hao and Trust, 2012; Ma et al., 2014; Fu, Shiau and Chang, 2014; Zhu, 2014; Wu and Shiau, 2015)**.

The variety of steamed bread technology can be combined according to such classification feature as a way of dough making into three main groups: sourdough, sponge and straight dough method.

The first method is usually used in domestic production and in small private enterprises. In this way, sourdough is added by 10% to the mass of flour, than a required amount of water is added and dough is mixed for 5 – 10 minutes. The fermentation lasts 3 hours at temperature of 26 – 28 °C and a relative humidity of 75%. Due to the presence of lactobacilli, dough pH is 3.7 – 4.0, so after fermentation 40% of Na_2CO_3 solution is used. To achieve the desired pH value of dough (6.4 – 6.7), on average, it is necessary to add a solution of Na_2CO_3 in an amount of 0.5% to the mass of flour. Neutralization of dough is critical in the production of steamed bread, since with excessive amounts of Na_2CO_3, color of products varies from yellow to dark gray, and the smell is expressed in alkaline. If the dough is not brought to the required pH, steamed bread has an acidic smell, low volume, unobtrusive appearance and excessively rigid structure. The introduction of an alkaline solution neutralizes acids that are released by lactobacilli when the dough precipitates, which helps to increase the release of carbon dioxide. Depending on the technology, the dough can be subjected to swirling after neutralization **(Li et al., 2012, Wu and Shiau, 2015)**. The finished dough is rolled and twisted into a long baffle and divided into pieces weighing 100 – 130 g, and then formed in oblong or rounded billets. Further, the dough pieces are subjected to proofing for 30 minutes at a temperature of 32 – 36 °C and relative humidity of 75 – 80% and steamed for 15 – 20 minutes **(Wu and Shiau, 2015)**.

Sponge dough method is widely used in steamed baking technology. The production process involves the following operations: sponge preparation, fermentation, dough kneading, rolling, forming, splitting into pieces, proofing and steaming. 80% of flour is mixed with yeast and water for sponge preparation. Yeasts are pressed or dried. After 60 minutes of fermentation at a temperature of 32 °C and a relative humidity of 80%, 20% of flour is added together with other ingredients **(Fu, Shiau and Chang, 2014; Li et al. 2015)**. There are two main advantages to using the sponge dough method. First, it is possible to develop a flexible fermentation schedule for different batches of bread; and secondly, the bread has a finely porous homogeneous structure of crumb and bright aroma. However, the sponge dough method requires higher labor costs, production space and a long process of production **(Fu, Shiau and Chang, 2014; Zhang et al., 2014)**.

Straight dough method is much simpler than previous one, because of simplicity and speed of the process, and therefore the most widespread. Straight dough method of steamed bread preparation is significantly shorter than the first two, but rather sensitive to duration change of technological process in industrial production. For example, if a technological scheme is not developed sufficiently, one batch of dough can achieve the optimum fermentation, while others will be overfermented or underfermented. In addition, bread will have less homogeneous structure and lower organoleptic and technological characteristics than bread produced by sponge dough method or using sourdough **(Kawamura-Konishi et al., 2013; Fu, Shiau and Chang, 2014)**.

The most important stage that results in the receipt of high quality products is bread proofing **(Meredith, 1965; Chen and Gan, 1997; Moayedallaie, Mirzaei and Paterson, 2010)**. The degree of proofing affects the shape, color, structure and aroma of finished products. Traditionally, proofing of the dough pieces is carried out at a temperature of 32 – 37 °C and a relative humidity of 70 – 80%. In most cases, the optimal duration of the process is determined visually by pressing on the surface of a piece. It is believed that optimally fermented dough pieces have shining and elastic surface that quickly restores its shape after pressing **(Meredith, 1965)**. However, this method of estimation lacks stability, so D. Chen **(Chen et al., 2010)** developed a standard method for evaluating the optimal duration of dough proofing. Under this method, 25 g of dough for the production of steamed bread is placed in a cylinder, diameter 3 cm and a volume of 45 mL. The amount of dough on average is 21 – 22 mL. After proofing, the final volume of dough for Southern steamed bread should be 38 mL, for Northern and Taiwan 45 mL **(Hou, 1991)**.

The final stage of production is the steamed treatment of dough pieces. The pieces are placed in an oven with steam convector and stem for 15 – 30 minutes at atmospheric pressure **(Sivaramakrishnan, Senge and Chattopadhyay, 2004)**.

The assessment of steamed bread quality is carried out by evaluation of the specific volume of products, organoleptic and consumer indicators. The standard specific volume of steamed bread of different types differs because of the differences in prescription composition. Southern steamed bread traditionally has a higher specific volume (average 3.5 – 3.7 $mL.g^{-1}$) than Northern (average 2.0 – 2.5 $mL.g^{-1}$) due to greater porosity. Steamed bread should have an elastic structure and quickly turn the shape on after pressing. High quality steamed bread should have a smooth and shiny surface of white color without yellow stains and symmetrical shape. Crumb of steam bread should be of white color, uniform fine porosity structure,

elastic and with high humidity **(Turabi, Sumnu and Sahin, 2008; Kim et al., 2009)**.

Increasing cases of celiac disease diagnosis, as well as low quality of available gluten-free products, prompts researchers to find new ingredients and technologies that can replace gluten and improve the properties of gluten-free bakery products.

Many scientists around the world are working on the development of gluten-free products. The main areas of development are the exclusion or modification of gluten from gluten-free raw materials and the complete exclusion of gluten-free raw materials from prescription mixtures. Relatively to the first direction, today research is conducted on the selection of gluten-free wheat, using genetic engineering **(Stoven, Murray and Marietta, 2012)**. However, the research data are under development. Therefore, the second direction of the solution of gluten-free products issue is more widespread. According to it, it is recommended to mix different types of gluten-free flour (mainly rice, corn, millet and buckwheat). The main problem of these technologies is to provide the structure of products, which is usually provided by gluten. In most cases, hydrocolloids (xanthan, gum, modified starches, etc.) **(Lobacheva, 2013; Medvid et al., 2017)**, enzymes (for example, transglutaminase) **(Shanina, Lobacheva and Gavrish, 2013; Lobacheva, 2015)** and sourdough **(Yeh et al., 2009)** are suggested to be used to solve this problem.

As a rule, gluten-free flour mixtures include four groups of food components: flour with high content of starch and non-starch polysaccharides; emulsifiers, dough fluffers, flavor ingredients; hydrocolloids; high protein ingredients **(Barsukova, Reshetnikov and Krasilnikov, 2011)**.

In our opinion, the most prospective enhancer of the bread structure made of rice flour (Frc) or corn flour (Fcn) is flour gluten-free raw materials with enriching action (FGFRM) – sorghum (Fsg), flaxseed (Ffs), quinoa (Fqn) and sunflower (Fsn) According to review of literary sources, the listed additives can improve structure of gluten-free products and their nutritional value due to the high content of complete proteins, as well as macro- and micronutrients. All of the given data relate to gluten-free bread, which is subject to baking, however, when steam is used for treatment, other thermal and biochemical processes can change the organoleptic, structural and mechanical properties of the final product, as well as its nutritional and biological value.

Scientific hypothesis

Conducted researches are aimed at determining the peculiarities of main stages behavior in gluten-free steamed bread production. Therefore, in order to achieve this goal, the primary task is to study the effect of FGFRM on the progress of main technological stages in steamed bread production, the structural and mechanical properties of dough, quality and consistency of final product.

MATERIAL AND METHODOLOGY

The following flour products are selected for experimental research and production testing:
-rice flour TM «Sto pudov»;
-corn flour TM «Sto pudov»;
-sorghum flour TM «Asparagus-LTD»;
-flaxseed defatted flour TM «Viva»;
-sunflower defatted flour TM «Efavit»;
-quinoa flour TM «Vivan».

For experimental studies flour is sieved through a laboratory nylon sieves with a hole size of 120 μm. The following ratio of components in flour mixes is selected:
-rice flour : flaxseed defatted flour (Frc:Ffs) – 95:5,
-rice flour : sunflower defatted flour (Frc:Fsn) – 95:5,
-corn flour : quinoa flour (Fcn:Fqn) – 85:15,
-corn flour : sorgum flour (Fcn:Fsg) – 90:10.

To obtain the liquid phase of dough, 1.75% of yeast is added to the mass of flour mixture in whole formulation of water (58 – 59% for bread based on rice flour and 63 – 64% for bread based on corn flour), salt is added in amount of 1.5% and stirred until a homogeneous solution is obtained. The flour mixture is moistened with a liquid phase to a given humidity and left for 25 – 30 minutes at a temperature of 30 – 35 °C for fermentation. After that, the dough pieces of 50 g are placed in baking pans pre-greased with sunflower oil, and left for 20 minutes at a temperature of 30 – 35 °C for proofing. Further, baking pans are placed in a laboratory steam oven TM «Moulinex» and subjected to steam treatment for 25 – 35 minutes. After that, bread is removed from baking pans and left at room temperature until it is completely drained.

Acidity of flour is determined by titrating water-flour suspension and aqueous-alcohol extract from the 0.1 N solution of NaOH in the presence of phenolphthalein **(Fedin, 1989)**. Rheological properties of dough are investigated using the Tolstoy elastoplastometer. Irreversible deformation, relative elasticity, plasticity and resilience are determined according to the standard method **(GOST, 1988)**.

Dough slackness is determined by **(Fedin, 1989)**. The titrated and active acidity of dough is determined according to **State standard DSTU 5024:2008** Total nitrogen of flour is determined by the Kjeldahl method **(GOST, 1987)**. Amount of protein is found by multiplying the content of total nitrogen by a conversion factor of 6.25. Gas-forming ability and rate of gas formation are determined in parallel with degree of dough loosening by the method **(Kuznetsova, 2010; Zapototska, Pichkur and Lysyj, 2013)**. Calculation of dry matters loss while fermentation is carried out in terms of glucose by the amount of CO_2 released during fermentation, using the fermentation Gay-Lusak equation for glucose **(Drobot, Mykhonic and Hryschenko, 2011)**.

Loss of carbon dioxide during fermentation is found by integration of area under fermentation curves. Changes in dough volume during fermentation are determined using 500 mL measuring cylinder, which contained 10 g of dough and kept at a temperature of 30 – 35 °C. Volume of dough is fixed every 60 seconds during 60 minutes. Water-retaining ability of dough is determined using a moisture balance **(Fedin, 1989)**.

Statistical analysis

Approximation of obtained experimental data was carried out using the least squares method, as well as MathCAD Prime 3.1 mathematical package and EXCEL 2016 spreadsheet packet, SPSS professional statistics version

17, statistical software Statistica 10.0. Degree of credibility for all experiment is 0.95.

RESULTS AND DISCUSSION

Dough mixing stage has a significant impact on the quality of finished products. During the mixing, structure of dough and subsequent structural and mechanical properties of products are formed. Dough has at the same time elastic-resilience and plastic-viscous properties. The phenomenon of resilience contributes to preservation of a given shape of products during formation, and elasticity allows to increase the productivity of dough kneading. Hydrocolloids addition significantly increased the gelatinization temperature (from 52.0 to 64.2 °C) and water absorption (from 56.22 to 66.50%) of dough **(Liu et al., 2018)**.

To evaluate elastic-resilience and plastic-viscous properties of dough for gluten-free steamed bread, study is conducted on a Tolstoy elastoplastometer. The humidity of the prototype samples is 45%.

Figure 1 shows the loading and unloading curves of gluten-free dough based on rice and corn flour. At the loading part of the curve, three areas can be distinguished – instantaneous resilience deformation, high elastic deformation, and a section of system flow. It can be noted that flour additives affect the resistance of rice flour dough and increase its strength. The effectiveness flaxseed and sunflower flour is not significantly different. The obtained results of corn dough research show similar tendencies. First of all, introduction of FGFRM significantly affects the reduction of irreversible relative deformation by 36 – 68%.

Introduction of flaxseed or sunflower flour contributes to an increase in relative elasticity of dough and to a decrease in resilience (Table 1). This result can be explained by high fat content in flaxseed or sunflower flour.

The results indicate that addition of FGFRM to corn dough in all cases contributes to increasing of its elasticity. This tendency is positive because of significant fragility of corn dough. At the same time, the relative plasticity decreases slightly by 16 – 18% and relative resilience increases (up to 2.3 times when using sorghum flour).

Obtained data correlates well with studies of dough slackness (Figure 2). It was established that for 3 hours of dough relaxation with addition of FGFRM slackness is 11 – 23% less than control samples which agrees with the reduction of irreversible relative deformation.

Thus, improvement of rheological characteristics of gluten-free dough based on rice or corn flour with addition of sorghum, flaxseed, quinoa and sunflower flour is experimentally proved. Therefore, these types of FGFRM can be considered as prospective in terms of structure formation of dough for gluten-free bread. According to research of rheological characteristics of dough, the introduction of FGFRM leads to improvement of dough resilience and elasticity, therefore, the increase of kneading duration, used in traditional technology of steamed bread, has no grounds. Thus, it is recommended to knead dough for 5 – 10 minutes.

The viscoelastic properties of the different doughs strongly influenced the bread volume and the crumb texture. Thus, starch-based breads showed higher specific volume and lower hardness during fermentation **(Martínez and Gómez, 2017)**.

The most important indicator of fermentation process efficiency is gas-forming ability, since this indicator directly affects the specific volume and porosity of final product. Change in dough acidity also has a great practical importance: with its increasing, the processes of protein swelling and peptization are intensified, which is accompanied by a change in their rheological properties **(Kuznetsova, 2010; Drobot, Mykhonic and Hryschenko, 2011)**. Active acidity of dough determines the presence of sour taste in bakery products, as well as intensity of enzymatic processes and influences on activity of microorganisms (in particular, yeast).

An important factor that determines baking properties of flour raw materials is intensity of dough fermentation. Use in gluten-free dough formulation FGFRM in order to regulate its technological properties significantly influences fermentation intensity and activity of amylolytic enzymes of flour (Figure 3 and Figure 4). It should be noted that fermentation of gluten-free dough is more intense than wheat, so the study is conducted for 100 minutes. Results show that addition of FGFRM leads to an increase in the amount of carbon dioxide in gluten-free dough by 10 – 30%. We believe that this dependence is due to the sufficient amount of sugars in FGFRM, especially flaxseed and sorghum, which can provide high quality products during technological process. In comparison with advanced technologies which include ultrasound treatment and increase this index only by 6.7% **(Luo et al., 2018)**, it is a significunt result.

On the basis of experimental data on flour gas-forming ability, speed of gas formation in dough was calculated (Figure 4).

In order to establish recommended dough fermentation regimes, study of dough volume changes is conducted. It is found that the addition of FGFRM slightly shifts the peak of fermentation process (Figure 5).

The results of research show that use of FGFRM leads to a slight slowdown in the process of dough fermentation. In most cases, accumulation peak of carbon dioxide is shifted by 10 minutes. In all cases of FGFRM use gas-retaining ability increases, which correlates with an increase in specific volume and porosity of bread.

Thus it is determined that the recommended duration of fermentation of dough based on rice flour with addition of flaxseed is 35 – 40 minutes, with the addition of sunflower 20 – 30 minutes, dough based on corn flour with addition of quinoa or sorghum 25 – 35 minutes.

Intensity of acid accumulation in dough is estimated by changing the active and titrated acidity parameters during fermentation. The results of experimental studies are presented in Figure 6 and Figure 7.

It should be noted that for the use of FGFRM indices of initial and final acidity of dough are different, however, the intensity of acid accumulation process has a similar nature.

Table 1 Rheological characteristics of gluten-free dough with introduction of FGFRM (degree of credibility α = 0.95).

Sample	Relative elasticity, Erel, %	Relative plasticity, Prel, %	Relative resilience, Rrel, %
Frc	3.56	84.51	11.93
Frc + Ffs	7.95	80.77	11.28
Frc + Fsn	11.72	83.45	4.83
Fcn	5.36	88.93	5.71
Fcn+ Fqn	21.17	72.77	6.06
Fcn + Fsg	12.73	74.09	13.18

Note: FGFRM – flour gluten-free raw materials with enriching action, Frc – dough from rice flour, Frc + Ffs – dough from mixture of rice flour and flaxseed defatted flour in ratio 95:5, Frc + Fsn – dough from mixture of rice flour and sunflower defatted flour in ratio 95:5, Fcn – dough from corn flour, Fcn:Fqn – dough from mixture of corn flour and quinoa flour in ratio 85:15, Fcn:Fsg – dough from mixture of corn flour and sorgum flour in ratio 90:10.

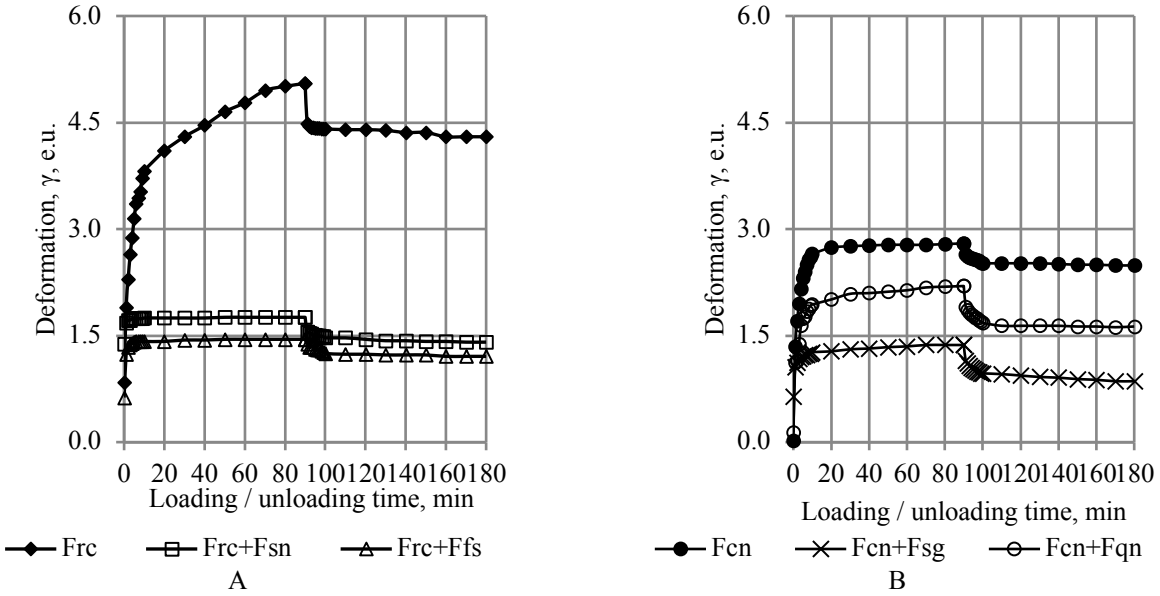


Figure 1 Loading and unloading curves based on rice (A) and corn (B) flour with introduction of FGFRM. Note: FGFRM – flour gluten-free raw materials with enriching action, Frc – dough from rice flour, Frc + Ffs – dough from mixture of rice flour and flaxseed defatted flour in ratio 95:5, Frc + Fsn – dough from mixture of rice flour and sunflower defatted flour in ratio 95:5, Fcn – dough from corn flour, Fcn:Fqn – dough from mixture of corn flour and quinoa flour in ratio 85:15, Fcn:Fsg – dough from mixture of corn flour and sorgum flour in ratio 90:10.

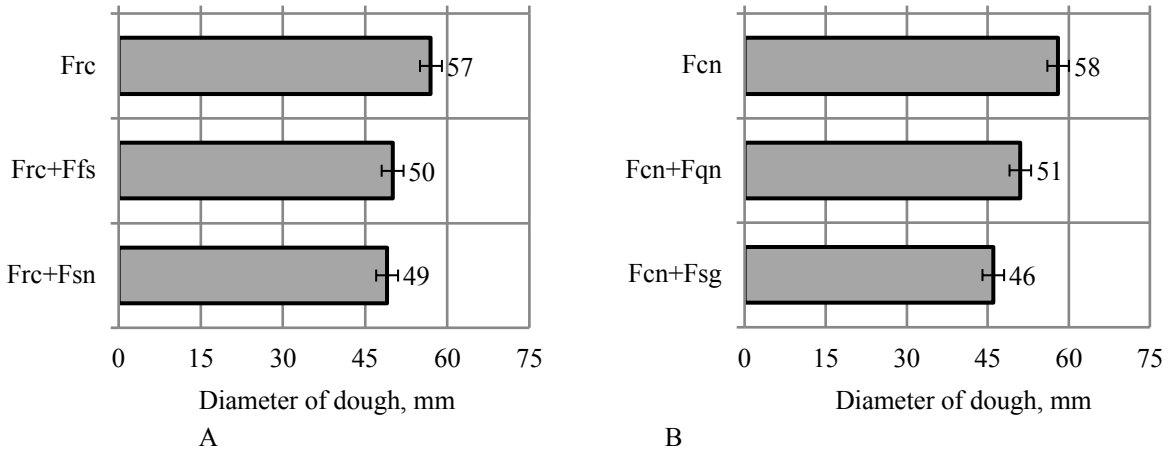


Figure 2 Influence of flour additives on dough slackness. Note: A – on the basis of rice flour, B – on the basis of corn flour.

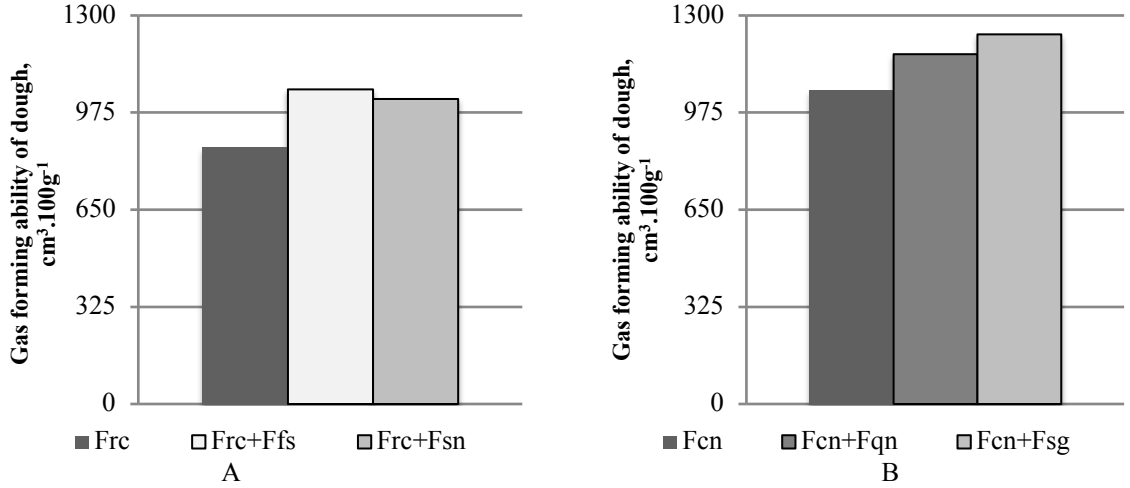


Figure 3 Change of gas forming ability of gluten-free dough on the basis of rice flour (A) and corn flour (B). Note: FGFRM – flour gluten-free raw materials with enriching action, Frc – dough from rice flour, Frc + Ffs – dough from mixture of rice flour and flaxseed defatted flour in ratio 95:5, Frc + Fsn – dough from mixture of rice flour and sunflower defatted flour in ratio 95:5, Fcn – dough from corn flour, Fcn:Fqn – dough from mixture of corn flour and quinoa flour in ratio 85:15, Fcn:Fsg – dough from mixture of corn flour and sorgum flour in ratio 90:10.

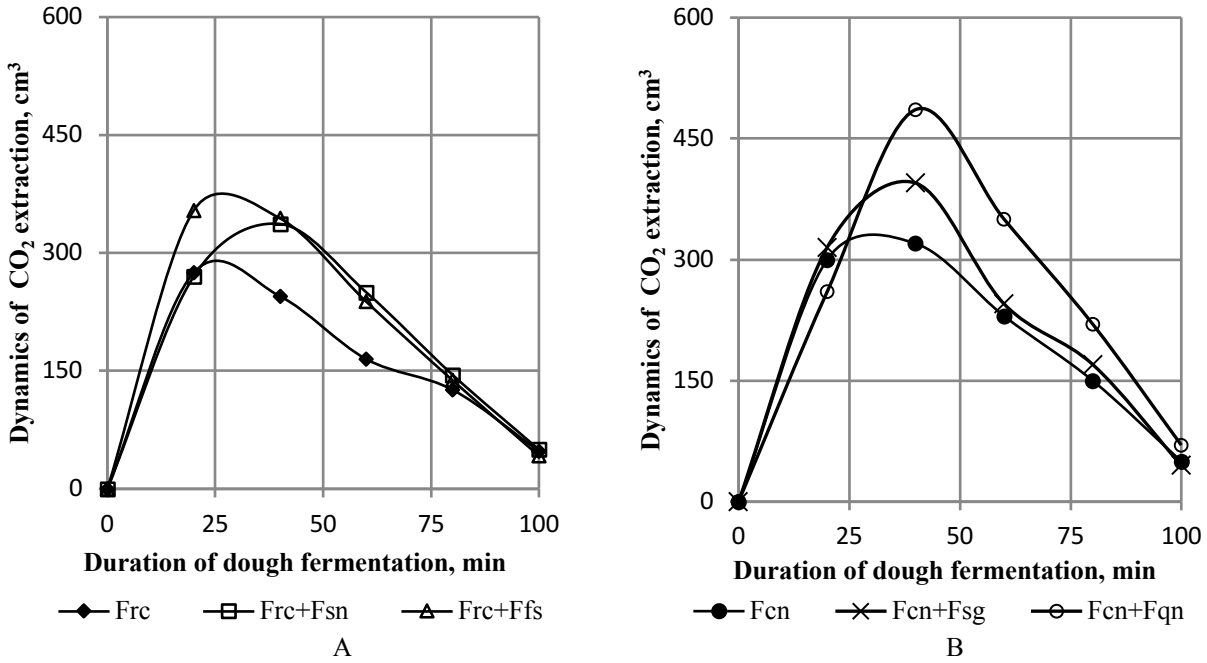


Figure 4 Kinetics of gas formation in gluten-free dough on the basis of rice flour (A) and corn flour (B). Note: FGFRM – flour gluten-free raw materials with enriching action, Frc – dough from rice flour, Frc + Ffs – dough from mixture of rice flour and flaxseed defatted flour in ratio 95:5, Frc + Fsn – dough from mixture of rice flour and sunflower defatted flour in ratio 95:5, Fcn – dough from corn flour, Fcn:Fqn – dough from mixture of corn flour and quinoa flour in ratio 85:15, Fcn:Fsg – dough from mixture of corn flour and sorgum flour in ratio 90:10.

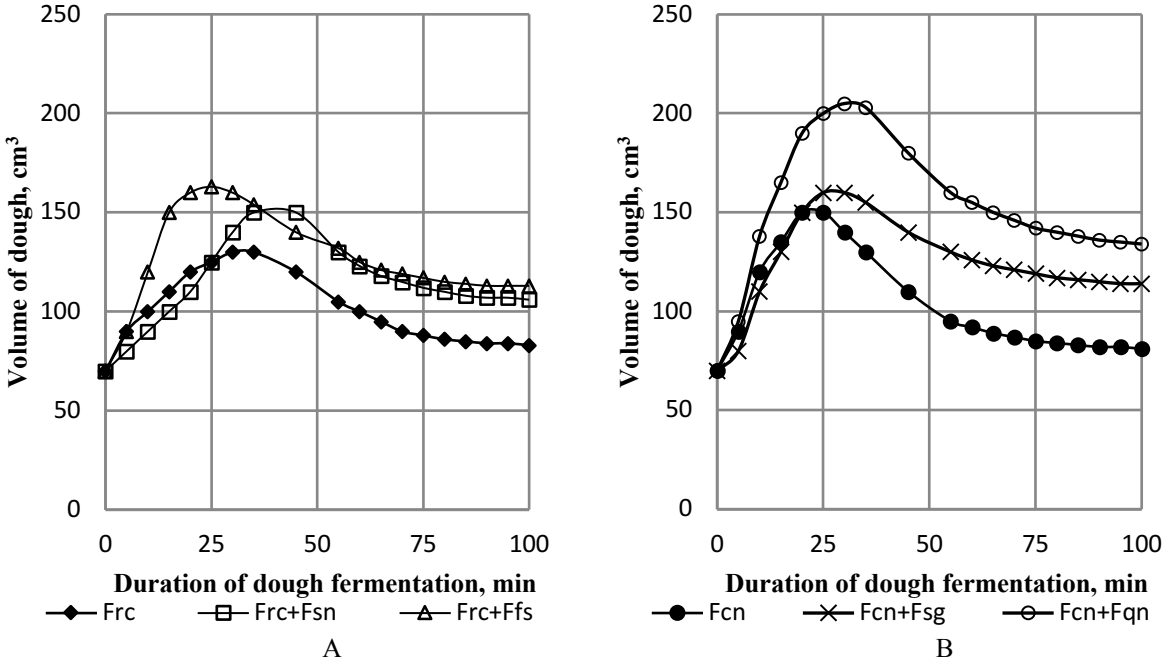


Figure 5 Change of volume of dough based on rice flour (A) and corn flour (B) during fermentation. Note: FGFRM – flour gluten-free raw materials with enriching action, Frc – dough from rice flour, Frc + Ffs – dough from mixture of rice flour and flaxseed defatted flour in ratio 95:5, Frc + Fsn – dough from mixture of rice flour and sunflower defatted flour in ratio 95:5, Fcn – dough from corn flour, Fcn:Fqn – dough from mixture of corn flour and quinoa flour in ratio 85:15, Fcn:Fsg – d ough from mixture of corn flour and sorgum flour in ratio 90:10.

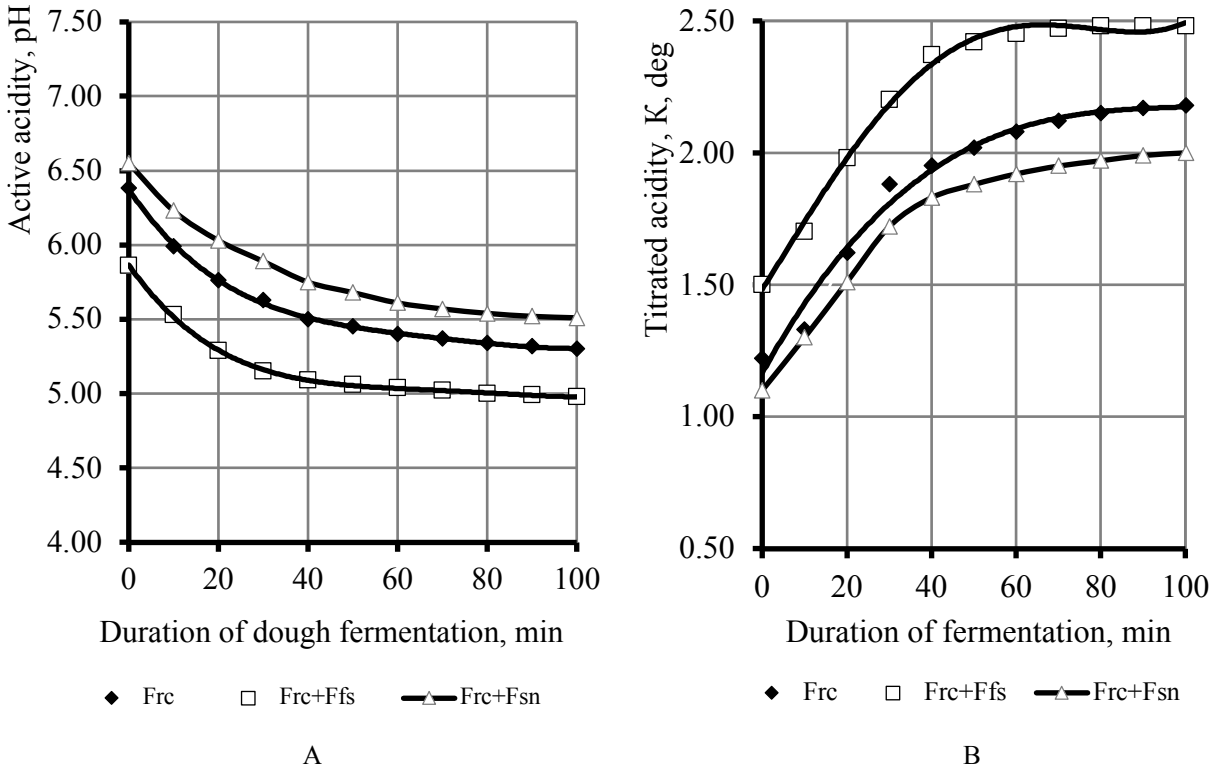
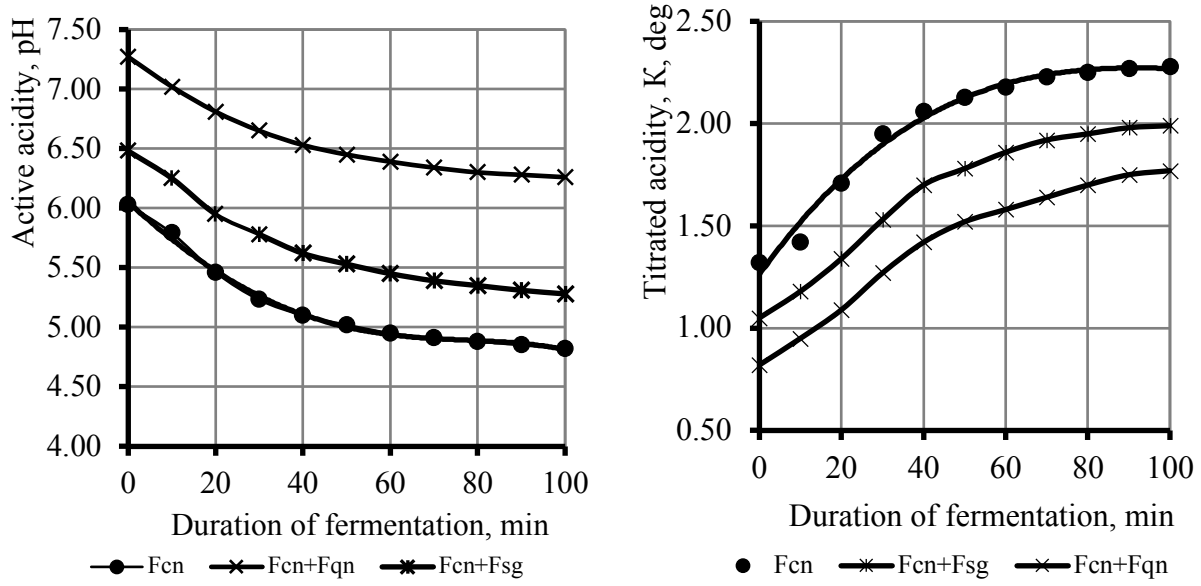


Figure 6 Change of active and titrated acidity of dough based on rice flour during fermentation. Note: FGFRM – flour gluten-free raw materials with enriching action, Frc – dough from rice flour, Frc + Ffs – dough from mixture of rice flour and flaxseed defatted flour in ratio 95:5, Frc + Fsn – dough from mixture of rice flour and sunflower defatted flour in ratio 95:5, Fcn – dough from corn flour, Fcn:Fqn – dough from mixture of corn flour and quinoa flour in ratio 85:15, Fcn:Fsg – dough from mixture of corn flour and sorgum flour in ratio 90:10.



A B

Figure 7 Change of active and titrated acidity of dough based on corn flour during fermentation. Note: FGFRM – flour gluten-free raw materials with enriching action, Frc – dough from rice flour, Frc + Ffs – dough from mixture of rice flour and flaxseed defatted flour in ratio 95:5, Frc + Fsn – dough from mixture of rice flour and sunflower defatted flour in ratio 95:5, Fcn – dough from corn flour, Fcn:Fqn – dough from mixture of corn flour and quinoa flour in ratio 85:15, Fcn:Fsg – dough from mixture of corn flour and sorgum flour in ratio 90:10.

Table 2 Quality of gluten-free steamed bread on the basis on the mixture of rice flour depending on durability of heat treatment (degree of credibility $\alpha = 0.95$).

Duration of stem treatment, min	Yield of bread, %	Quality characteristics of bread			
		Crust characteristics	Crumb characteristics	Aroma	Taste
on the basis of rice and flaxseed flour in the rate 95:5					
20	199.3	sticky, particularly separates from baking pan	sticky, not thoroughly baked	yeast smell of unbaked dough	taste of unbaked dough
25	202.9	sticky, completely separates from baking pan	sticky inside, thoroughly baked closer to surface		taste of not thoroughly baked dough
30	204.2	moderately sticky	thoroughly baked, non-sticky	specific for rice bread	specific for rice bread
35	205.6				
40	207.3				
on the basis of rice and sunflower flour in the rate 95:5					
20	195.4	sticky, particularly separates from baking pan	sticky, viscous, unbaked	yeast smell of unbaked dough	taste of unbaked dough
25	198.8	insignificantly sticky	sticky, not thoroughly baked		taste of not thoroughly baked dough
30	200.5				
35	201.2	non-sticky	thoroughly baked, non-sticky	specific for rice bread	specific for rice bread

Table 3 Quality of gluten-free steamed bread on the basis on the mixture of rice flour depending on durability of heat treatment (degree of credibility α = 0.95).

Duration of stem treatment, min	Yield of bread, %	Quality characteristics of bread			
		Crust characteristics	Crumb characteristics	Aroma	Taste
on the basis of corn and sorghum flour in the rate 90:10					
20	203.5	sticky, completely separates from baking pan	sticky, viscous, unbaked	yeast smell of unbaked dough	taste of unbaked dough
25	204.4	insignificantly sticky	sticky, not thoroughly baked		
30	205.2	non-sticky	sticky, thoroughly baked	specific for corn bread	specific for corn bread
35	205.9				
40	206.0		thoroughly baked, non-sticky		
on the basis of corn and quinoa flour in the rate 85:15					
20	203.7	sticky, completely separates from baking pan	sticky, viscous, unbaked	yeast smell of unbaked dough	taste of unbaked dough
25	204.0	insignificantly sticky	sticky, not thoroughly baked		
30	205.8	non-sticky	thoroughly baked, non-sticky	specific for corn bread	specific for corn bread
35	206.2				
40	206.9				

It is noteworthy that flaxseed flour reduces active acidity of dough, and sunflower, on the contrary, increases this index. For 100 minutes of fermentation, the index of titrated acidity varies for a sample made from rice flour from 1.22 to 2.18 °H (a difference is 0.96 degrees) in sample Frc + Ffs – from 1.5 to 2.0 °H (difference is 0.50 degrees) in the sample Frc + Fsn – from 1.1 to 2.0 °N (difference is 0.9 degrees).

Change in active acidity of dough based on rice flour during fermentation is also identical. Thus, for 100 minutes of fermentation, the sample Frc has a pH value of 5.3, sample Frc + Ffs – 4.98, sample Frc + Fsn – 5.51.

Reducing pH value when we add flaxseed flour and higher values of the initial acidity of the sample Frc + Ffs are due to the accumulation of oxidation products of fats contained in this raw material.

Thus, according to the research complex, it can be concluded that dough with addition of FGFRM in the process of fermentation can provide necessary level of microbiological and enzymatic processes for obtaining bakery products with organoleptic properties of high quality.

Although the production of gluten free bread still remains a technological challenge, research continues to find innovative approaches to improve the quality of gluten free bread. Literature shows that an important aim is to imitate the gluten-network by combining several ingredients, from which hydrocolloids play a crucial role. Also crosslinking enzymes have been increasingly investigated. On the other hand, a carbohydrate network formed by arabinoxylans offers an innovative and more natural approach for improving gluten free products **(Wang, Guo and Zhu, 2016; Bender and Schönlechner, 2020)**.

In production of gluten-free steamed bread baking process is replaced by steam treatment to prevent formation of acrylamides and other carcinogens and preserve food and biological value of final product. Steam treatment is more gentle mode, which is significantly different from traditional baking. When the processing time in the steam chamber increases, the quality is almost unchanged, but the cost is increased. Therefore, to assess the quality of steamed bread, it is important to find the value of heat treatment duration, in which film-like crust is formed on surface of products, the starch is gelatinized, proteins are denaturated, crumb loses excessive

adhesiveness and the products acquire good consumer properties.

Taking into account the foregoing, to determine the duration of heat treatment by steam, adhesiveness of surface of gluten-free bread, smell, taste and yield of finished products are determined with addition of FGFRM (Table 2 and Table 3).

Steam treatment of gluten-free steamed breads based on a mixture of rice and flaxseed flour for 20 minutes results in a sticky texture of the crumb and surface. Products can not be completely separated from baking pads. After 25 minutes after the start of heat treatment bread is well separated from baking pads, but there is increased adhesiveness in the middle of crumb. 30 minutes later bread is well baked throughout the volume, but still there is a yeast smell and taste of raw dough. Thus, recommended durability of heat treatment with steam of bread based on rice and flaxseed flour is in a ratio of 95:5, which is 35 minutes.

With an increase of steam treatment duration, the yield of finished products is slightly increased, which can be explained by moisture binding by biopolymers of flour raw materials. A similar trend is observed in the sample based on rice and sunflower flour in the ratio of 95:5. Study of gluten-free steamed breads based on corn and sorghum flour in the ratio of 90:10 and corn and quinoa flour in the ratio of 85:15 shows that good consistency of the crumb and surface can be achieved with steam treatment duration for 30 minutes. In such conditions, the smell and taste of bread corresponds to ready products. Recipes and methodologies are grouped by (main) starch source and list other ingredients, additives and treatments used **(Masure, Fierens and Delcour, 2016)**. Additional ingredients significantly change quality of steamed bread. Thus, implementation of inuline, fresh steamed bread gained the highest score, possessing a lighter color, higher specific volume and softer texture **(Kou et al., 2019)**. Use of bran is also able to rise quality of steamed bread, improving surface smoothness, crumb structure and stress relaxation scores **(Ma, Lee and Baik, 2018)**.

The results confirm that it is possible to reach the full readiness of bread based on rice flour after 35 minutes of steam treatment, and on the basis of corn flour after 30 minutes.

According to the results of experimental studies, it is established that the technological stages of gluten-free steamed bread production do not undergo significant changes. A new kind of equipment is the steamer that is installed to replace the oven. Product is prepared in a single-phase method, adopted in practice of baking bread. The duration of dough mixing is 10 – 15 min, fermentation 20 – 35 min. Steam treatment is carried out under atmospheric pressure.

FGFRM is supplied to the plant bakery by flour tracks. Flour from cistern of a flour track is loaded into silos for storage under pressure through pipes and filter. Additional raw materials (salt, sugar) are dissolved and sent for storage in containers. Preparation of other raw materials for production is carried out in accordance with "Technological instructions for the production of bread and bakery products". Yeasts are diluted with water in containers and sent to a dosing station for liquid components, followed by dough preparation. When working on the line, gluten-free flour from the silos is dispensed and supplied to scales automatically. Then flour enters the intake filter. The flour is cleaned from impurities on a screen with a magnetic trap. Next, a gluten-free flour mixture is formed. A better usage of cereal by-products as valuable ingredients in foods would aid the economics and the sustainability of cereal chain **(Čukelj Mustač et al., 2020)**. Components of mixture are weighed on automatic scales, mixed in a screw mixer and loaded into silos. To knead dough, gluten-free flour is weighed and sent to a dough machine. Additional raw materials (solutions of salt, sugar, yeast) are sent to dough machine from containers, through the station for liquid components dispensing. Kneaded and fermented dough is sent to dough separator, with help of which portions of the dough of the same mass are received. After this, the manipulator, using a dividing table, stack the dough pieces of a certain mass and form into proofing cabinet. Proofing of dough pieces is carried out during 10 – 15 min at temperature of 30 – 32 °C and relative humidity of 65 – 70%. Dough pieces after proofing are put to the steamer for steam treatment. Products are treated with steam for 30 – 35 min at a temperature of 92 – 97 °C. Finished products get to the circulation table by drain device gutter of finished product, a stacker loads them to a container. Trays with products are loaded to truck. Gluten-free steamed bread can be packed in different ways, for example there is effective technology with use of vacuum degree **(Xu et al., 2020)**.

CONCLUSION

Obtained results show that addition of FGFRM leads to a change in the main stages behavior of gluten-free steamed bread production.

Use of FGFRM leads to a decrease in irreversible relative deformation of dough for 36 – 68% and to increase of its elasticity. At the same time, the relative plasticity decreases by 16 – 18% and the relative resilience increases (up to 2.3 times when using sorghum flour). The recommended duration of dough mixing is 10 – 15 min.

In the presence of FGFRM, the amount of carbon dioxide accumulated in gluten-free dough increases by 10 – 30%.

Recommended duration of fermentation of rice flour dough with the addition of flaxseed is 35 – 40 minutes, with the addition of sunflower 20 – 30 minutes, for dough based on corn flour with the addition of quinoa or sorghum 25 – 35 min.

Use of FGFRM does not affect the intensity of acid accumulation process. It is established that flaxseed flour reduces active acidity of dough, and sunflower, sorghum and quinoa flour, on the contrary, increases this index.

Recommended steam processing time is 35 min for bread based on rice flour, 30 min for based on corn flour.

Technological scheme of production of gluten-free steamed bread is offered. Compared to the traditional one, it is recommended to use a steamer instead of oven.

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EXTRACTIVE STRIPPING VOLTAMMETRY AT A GLASSY CARBON PASTE ELECTRODE FOR ANALYSIS OF COW'S MILK AND CREAM

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ABSTRACT

In this paper, a procedure based on extractive accumulation of milk fat globules (MFGs) into a pasting liquid (lipophilic binder) of glassy carbon paste electrode (GCPE) with subsequent electrochemical detection by square-wave voltammetry (SWV) in 0.1 mol L^{-1} Britton-Robinson buffer of pH 4.0 has been tested to find out whether it can be utilized as a simple screening analytical method for cow's milk and cream nutrition control. Since there is assumption that the necessary alkaline hydrolysis of cow's milk and subsequent extraction of lipophilic vitamins into an organic solvent could be avoided, several GCPEs differing in type (atactic polypropylene, paraffin oil, paraffin wax, silicone oil, and vaseline) and content (5, 10, 15, 20, and 25%; w/w) of pasting liquid used were tested as part of complex optimization. The obtained results show that MFGs contain predominantly vitamin A (carotenoids and retinoids), especially all-*trans*-retinol, which could serve as significant marker of the fat content. However, their individual forms were not possible to distinguish due to the considerable anodic peak broadening (overlapping).

Keywords: carbon paste electrode; cow's milk; extraction; milk fortification; nutrition control; voltammetry

INTRODUCTION

In the mammary glands, milk fat globules (MFGs), ranging in size from 0.1 to 15 μm in diameter **(Logan et al., 2014)**, originate as fat droplets composed largely (>98%) of triacylglycerols (TCGs). These fat droplets are evenly emulsified throughout the volume and contain lipophilic (fat-soluble) vitamins dissolved in them **(Heid and Keenan, 2005)**. Losses of naturally occurring lipophilic vitamins are significant after mechanical separating the milk fat (cream) from the raw milk. Obtained skimmed milk is then homogenized that is a process of breaking down the large fat droplets under high pressure so that they stay together and do not separate as cream. To improve the nutritional values, the homogenized milk is usually fortified by extra vitamins (retinyl palmitate and cholecalciferol) and minerals that are not naturally found in milk in significant amounts **(Trinidad et al., 2015)**.

The cow's milk and products made from it are considered as very complex sample matrixes and their analysis is often complicated and time-consuming **(Trenerry et al., 2011)**. Valid reference analytical methods used for lipophilic vitamins determination in foodstuffs in laboratories of the Czech Agriculture and Food Inspection Authority 211/2004 Coll4. utilize a HPLC with UV detection, known as standard: ČSN EN 12823 (vitamin A), ČSN EN 12821 (vitamin D), ČSN EN 12822 (vitamin E) and ČSN EN 14148 (vitamin K). In addition, a gravimetric method (EN 1211) is used to determine milk fat content.

Evaluation of the lipophilic vitamins content in milk (also dairy produce) has its substantiation, especially in case of human nutrition which deals on provision of essential nutrients in food necessary to support human life and health **(Haug et al., 2007)**. Moreover, analytical methods for simultaneous determination of lipophilic vitamins and their provitamins in milk using microcolumn **(Gomis et al., 2000)**, narrow-bore column **(Blanco et al., 2000)** and two-dimensional liquid chromatography **(Zhang et al., 2015)** have been developed.

Time-consuming sample preparation is the most challenging step in the analysis as it involves several steps (alkaline hydrolysis, liquid-liquid extraction, filtration and evaporation of organic solvent) in which the analytes may be lost **(Trenerry et al., 2011)**. To avoid degradation of analytes, the alkaline hydrolysis shoud be carried out in presence of an antioxidant, under an inert atmosphere, and in absence of light.

A simple semiquantitative method for the determination of vitamin D in skim milk is worth mentioning **(Michlová et al., 2012)** when a sample is diluted with water, ethanol, and an aqueous ammonia solution. Vitamin D is subsequently extracted with a mixture of ether and hexane

for 4 hours. After evaporation of the organic solvent, vitamin D is transferred to the appropriate solvent (usually methanol or acetronitrile). The advantage of this procedure is that the sample does not need to undergo alkaline hydrolysis.

This paper offers a simple screening voltammetric method for monitoring vitamin A content (sum of retinoids and carotenoids) in cow's milk and cream samples without the need for a use complicated sample preparation. Since all lipophilic vitamins are electrochemically active organic compounds that undergo oxidation (vitamins A, D and E) or reduction (vitamin K) electrode reactions **(Lovander et al., 2018)**, they can be directly extracted from the milk into a pasting liquid (nonpolar binder) of glassy carbon paste electrode (GCPE). After transferring GCPE into an aqueous working medium (medium−exchange approach), the electrochemical detection of accumulated vitamins can be performed using a pulse voltammetric technique **(Sýs et al., 2019)**, namely square-wave voltammetry (SWV).

Scientific hypothesis

In this work, an effort was to find out whether square-wave anodic stripping voltammetry at GCPE can represent a suitable method for rapid determination of vitamin A.

MATERIAL AND METHODOLOGY

Chemicals and reagents

All-*trans*-retinol (≥95%) and ethanol (≥99.5%) were purchased from Sigma-Aldrich. Universal 0.1 mol.L^{-1} Britton-Robinson buffer (BRB), prepared by mixing of appropriate amounts of boric acid, glacial acetic acid, 85% phosphoric acid, and sodium hydroxide all from the aforementioned company, was used in selection of suitable detection medium. BRB was prepared using deionized water (minimum electric resistivity 18.2 MΩ cm, maximum 3 µg L^{-1} of total organic carbon) made in a Milli-Q® ultrapure water system from Merck Millipore (Burlington, USA).

Instrumentation

Voltammetric detection of accumulated lipophilic vitamins into the pasting liquid was performed in a conventional three-electrode arrangement containing always GCPE (working), silver chloride electrode with 3 mol.L^{-1} KCl salt-bridge (reference) and platinum sheet (auxiliary electrode). These electrodes were connected to the potentiostat Autolab PGSTAT101 controlled by software Nova (Version 1.11.0), both from Metrohm (Prague, Česká republika).

Preparation of carbon paste electrode

Glassy carbon powder of type Sigradur G (mixture 5 – 20 µm, HTW Maintingen, Germany) and one of randomly selected pasting liquids were mixed in a ceramic mortar for 15 min to create homogenous glassy carbon paste. The amount of tested pasting liquid differed from 5 to 30% (w/w). The resulting glassy carbon paste was packed into the cavity of the Teflon® piston-driven electrode holder with an end-hole of 3 mm in diameter. It is necessary to mention that the height of column in the cavity must be less than 2 cm due to difficult extrusion of glassy carbon paste. It is recommended that freshly prepared GCPEs should not be employed in any experiments due to their rather unstable electrochemical behaviour attributed to an incomplete homogenization. Consequently, freshly prepared GCPEs were left at the laboratory conditions for one day. After this self-homogenization process, GCPEs can be used for following voltammetric measurements **(Sýs et al., 2017)**.

Methods

Principle of medium-exchange extractive stripping voltammetry is illustarted in Figure 1. The extraction of lipophilic vitamins into the pasting liquid was carried out from 10 mL non-treated milk and cream samples (available in Czech stores) without need to apply a potential in the electrode cell (nonelectrolytic preconcentration), which is an approach known as "open circle procedure". After 10 min, GCPE enriched with analytes was rinsed with a stream of deionized water and immersed together with others electrodes into 0.1 mol.L^{-1} BRB. Final voltammetric detection was performed using square-wave voltammetry at potential range from 0 to +1.4 V, potential step (E_{step}) of 5 mV, potential amplitude (E_{ampl}) of 25 mV and frequency (f) of 50 Hz.

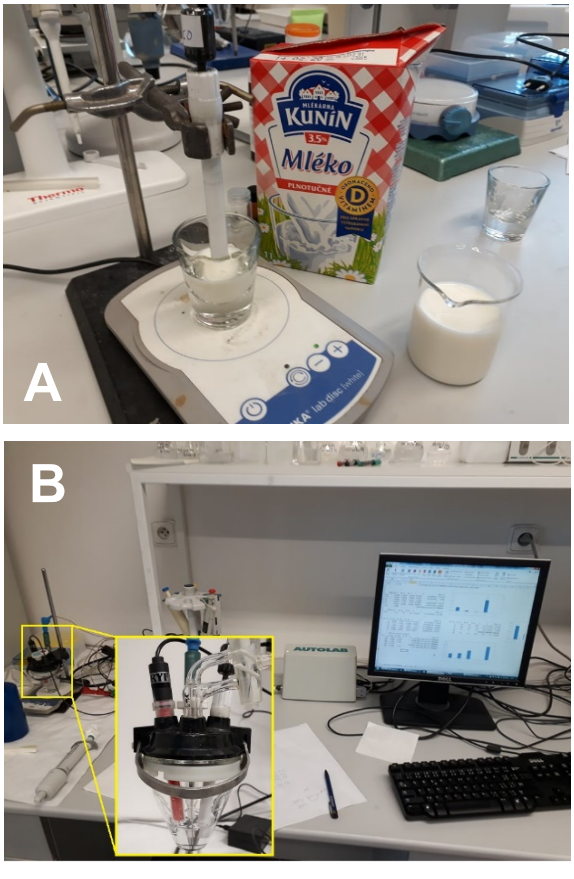

Figure 1 Individual steps of extractive stripping voltammetry with medium-exchange (A; accumulation and B; electrochemical detection using SWV).

Statistic analysis

Extraction repeatability

Generally, the repeability may be expressed by several indexes, nemaly coefficient of repeatability (CR), coefficient of variation (CV) and intra-class correlation

coefficient (ICC). CR defined by formula (1) is a precision measure which represents the value below which the absolute difference between two repeated test results may be expected to lie with a probability of 95%. The standard deviation (σ) under repeatability conditions is part of precision and accuracy.

$$CR = 1.96\sqrt{2\sigma^2} \quad (1)$$

However, the repeatability is more often given by CV defined as the ratio of the standard deviation (σ) and to the mean (μ). If this ratio is expressed as a percentage (see Eq. 2) then it will be referred to as relative standard deviation (RSD).

$$RSD = \frac{\sigma}{\mu} * 100 \quad (2)$$

Sufficient extraction repeatability constitutes the main criterion for development of voltammetric methods utilizing extractive accumulation to be able to use them for analytical purposes. ICC could not be used because units of two physical quantities (variables) were statistically tested only. Therefore, using RSD can be probably expected to be sufficient.

RESULTS AND DISCUSSION

Selection of pasting liquid type

Several GCPEs differing in the type of pasting liquid and containing always 20% (w/w) portion were investigated in SWASV of cow's milk (3.5% fat) to choose the optimum one. Working conditions for this experiment were as follows: accumulation for 10 min, stirring at 400 rpm, electrochemical detection in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{start} = 0 V, E_{end} = +1.4 V, E_{step} = 5 mV, E_{ampl} = 25 mV and f = 10 Hz. Due to relatively high current response and required reproducibility (Table 1), silicone oil should be taken for optimum extraction of lipophilic vitamins.

Table 1 Comparison of glassy carbon paste electrodes.

Pasting liquid	R (Ω)	E_p (V)	I_p (μA)
Atactic polypropylene	10.5 ±0.8	0.831	0.075
Paraffin oil	7.1 ±0.2	0.851	3.69 ±1.6
Paraffin wax	4.7 ±0.3	0.836	0.24 ±0.1
Silicone oil (8000 cSt)	8.0 ±0.2	0.844	1.14 ±0.2
Vaseline	17.4 ±1.0	0.829	0.013

Note: Values (R; ohmic resistance; E_p; peak potential, I_p; peak current response) given as $\mu \pm 2\sigma$ (95% probability) for five repetitions.

Ratio between carbon powder and pasting liquid

Under the prediction, an amount of extracted lipophilic vitamins would increase with a higher content of paste liquid in GCPE. However, electrochemical properties of GCPE are affected by ratio between glassy carbon powder and paste liquid. In principle, it can be stated that carbon particles remain in intimate contact (electrically conductive) until the amount of paste liquid exceeds 30% (w/w) **(Švancara and Schachl, 1999)**.

Surprisingly, it was found that the highest peak current response (unfortunately, background current (I_b) as well) was obtained at GCPE containing 5% (w/w) silicone oil, as demonstrated in Table 2. Despite high current yield, the content of 15% (w/w) silicone oil was chosen as optimum, thanks to the high reproducibility of accumulation. A non-specific adsorption of milk fat onto electrode surface, which acts as an electrical insulator causing a significant increase of background current (baseline signal), can be considered as possible explanation.

Table 2 Effect of silicone oil content in GCPE.

Content (%)	R (Ω)	I_p (μA)	I_b (μA)
5	6.7 ±0.2	10.0 ±0.3	66.4 ±3.50
10	7.0 ±0.3	4.7 ±0.3	8.9 ±0.90
15	4.8 ±0.2	5.1 ±0.3	4.4 ±0.50
20	8.0 ±0.2	1.1 ±0.2	0.9 ±0.02
25	6.0 ±0.1	0.9 ±0.1	0.7 ±0.02

Note: Values (R; ohmic resistance; I_p; peak current response; I_b; background current) given as $\mu \pm 2\sigma$ (95% probability) for five repetitions.

Effect of accumulation time

Principally, the optimum value of accumulation time is defined as a period required for reaching the equilibrium of lipophilic vitamins distribution between a nonopolar pasting liquid of GCPE and used milk sample. The cow's milk can be considered as a direct emulsion (so-called the first type emulsion) because a small amount of fat droplets (organic phase) are uniformly distributed throughout the milk volume (aqueous phase).

Resulting saturation curve describing dependence of current peak height on accumulation time showed a typical extraction equilibrium isotherm, as shown in Figure 2. The extraction equilibrium has been achieved after 600 s because using accumulation for longer period did not cause any significant increase in peak current response. Hence, accumulation time of 10 min was chosen as optimum.

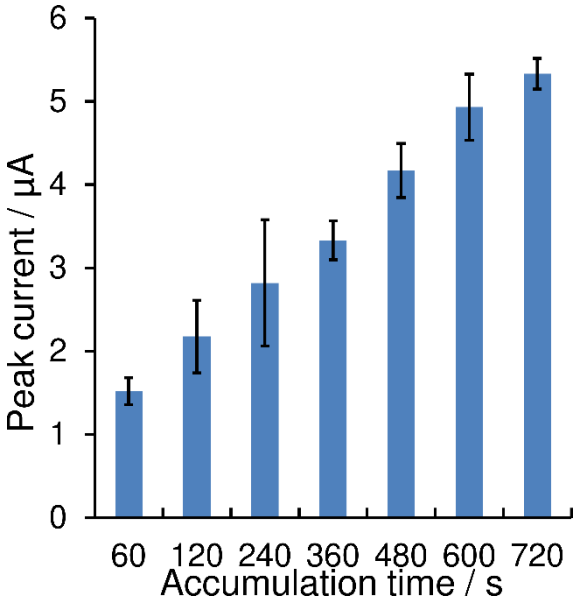

Figure 2 Effect of extraction time on extraction yield of lipophilic vitamins from the cow's milk (3.5% fat).
Note: Data obtained from SWV at GCPE containing 15% (w/w) silicon oil, E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 20 Hz.

Effect of stirring speed

Stirring speed affects the rate of fat droplets transport to the electrode surface where these droples containing lipophilic vitamis are then extracted into the pasting liquid of GCPE. Under this study, it was found that setting the rate of magnetic stir bar higher than 300 rpm did not have any significant effect on increase in final peak current response. Therefore, the above mentioned value can be considered as an optimum for subsequent experiments.

Identification of lipophilic vitamins in cow's milks

The fat dispersed in cow's milks and creams is formed by non-polar TCGs that are surrounded by phospholipids and membrane lipoproteins **(Heid and Keenan, 2005)**. At the natural pH of the cow's milk, they carry a negative charge and thus prevent bonding of MFGs. It is worth considering whether whole MFGs are extracted into the pasting liquid or a further equilibrium distribution of present lipophilic vitamins between the TCGs and the pasting liquid cannot exist. It can be assumed that both processes take place simultaneously. TCGs extracted (adsorbed) block surface of GCPE and therefore cause a dramatic increasing the background current.

From the literature **(Indyk and Woollard, 1997; Hulshof et al., 2006; Trenerry et al., 2011; Musara and Nyagura, 2017)**, lipophilic vitamins are found primarily in the milk fat. Unlike cholecalciferol (vitamin D3) and phylloquinone (vitamin K1) present in limit amounts (0.1 µg per 100 g), α-tocopherol (vitamin E), and retinol together with its provitamines (carotenoids) such as β-carotene, zeaxanthine and luteine (vitamin A) are the major representatives (40-110 µg per 100 g). Moreover, an artificially added retinyl palmitate **(Jensen et al., 1991)** can be present as well.

Generally, most extracted lipophilic vitamins and their provitamins usually provide very broad sensitive oxidation/reduction peaks (up to 250 mV) due to slow kinetic of corresponding electrode reactions occurred at liquid-liquid interface **(Sýs et al., 2019)**. As confirmation, a broad anodic peak beginning +0.705 V at and ending at +1.007 V was obtained for all investigated cow's milks and creams.

It is therefore impossible to distinguish and determine the individual forms of retinoids and carotenoids **(Žabčíková et al., 2018)**. Nevertheless, a number of published scientific papers suggest that the all-*trans*-retinol occupies a dominant position **(Jensen, 1994; Hulshof et al., 2006; Hodulová et al., 2015)**. Thus, it can be assumed that the peak obtained most likely corresponds to the anodic oxidation of all-*trans*-retinol a +0.852 V (compare with (overlapping peak at +0.886 V for cow's milk), as shown in Figure 3.

It seems that proposed extractive stripping voltammetry (ExSV) based on direct immersing of GCPE into continuously stirred cow's milk (3.5% fat) and subsequent electrochemical detection using SWV provides the desired sensitivity for detecting the sum of retinoids and carotenoids. A quantitative or at least semi-quantitative determination of vitamin A in cow's milk and cream samples was not the aim of this study. Voltammetric analysis of cow's milk enriched by differently defined amounts of all-*trans*-retinol could be probably considered as semi-quantitative analytical method.

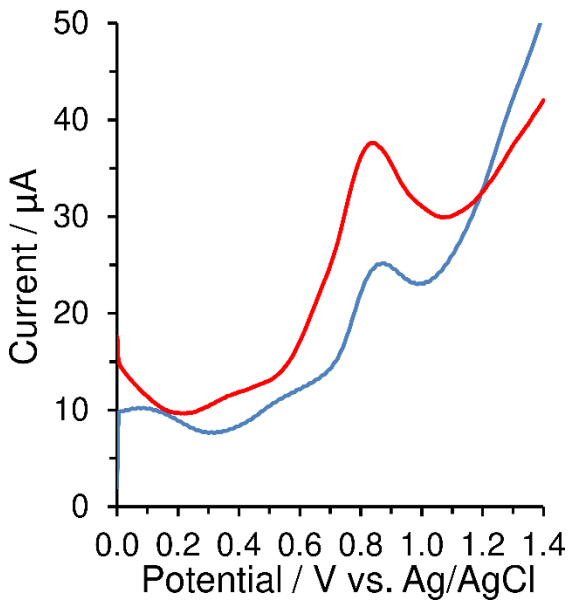

Figure 3 SWV voltammogram.
Note: SWV voltammogram of extracted (at 400 rpm for 10 min) cow's milk (3.5% fat) into GCPE containing always 15% (w/w) silicone oil with subsequent voltammetric detection in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 50 Hz (blue). SWV voltammogram of all-*trans*-retinol extracted (at 400 rpm for 5 min) from its (500 µmol.L^{-1}) 60% ethanolic solutions into GCPE containing always 20% (w/w) silicone oil with subsequent voltammetric detection in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{step} = 1 mV, E_{ampl} = 25 mV, and f = 25 Hz (red line).

Selection of detection medium and supporting electrolyte

Optimisation consisted in finding out proper working conditions for an anodic oxidation of lipophilic vitamins which are presented in MFGs accumulated into silicone oil. At first, the electrochemical detection has been subjected to pH study which was investigated for 0.1 mol.L^{-1} RBRs of pH values from 2 to 7. A linear relationship between peak potential and pH values of used supporting electrolytes, statistically evaluated as E_p = -0.0558 pH + 1.0891 (R^2 = 0.9978), was observed. The peak potential was shifted to more negative values with increased pH of used BRBs. This phenomenon probably occurs due to lowering the energy barrier and easier deprotonation of present lipophilic vitamins. The value of slope 0.0558 indicates the transition of electrons together with protons in a 1:1 ratio. The most sensitive peak current response was achieved using BRBs of pH values 4 and 5. It seems that BRB would be replaced by an acetate buffer of pH 4.5, more simple in composition.

Optimization of square-wave voltammetry

Parameters of SWV, potential amplitude and frequency, were optimised at constant potential step of 5 mV. Extracted lipophilic vitamins provided a broad anodic peak which height increased with increasing potential amplitude up to value of 25 mV. Therefore, potential amplitude of 25 mV was chosen for following analysis of cow's milks and creams. As shown in Figure 4, the height of anodic peak linearly increased with higher frequency. However, it

was observed that background current (baseline) increased as well. Hence, a value of 50 Hz representing a compromise was chosen as optimum.

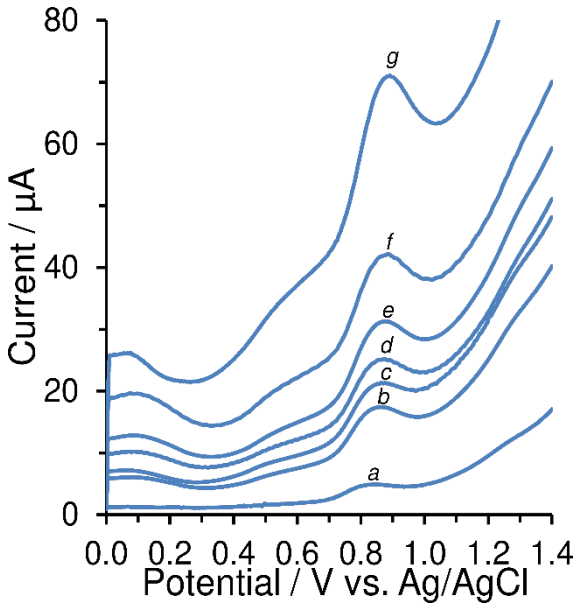

Figure 4 Voltammograms of lipophilic vitamins.
Note: Voltammograms of lipophilic vitamins sum (predominantly all-*trans*-retinol and β-carotene) extracted from cow's milk (3.5% fat) in GCPE containg 15% (w/w) silicone oil at 300 rpm and for 10 min. After rinsing with distilled water, subsequent voltammetric detection was performed in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 10 (*a*), 20 (*b*), 30 (*c*), 40 (*d*), 50 (*e*), 60 (*f*) and 100 (*g*) Hz.

Analysis* of *cow's milks and creams

At first, it is necessary to mention that a sample of whipped cream (40% fat content) could not be analysed using designed protocol due to high viscosity **(Van Vliet and Walstra, 1980)**. The whipped cream completely covered GCPE used and did not allow rinsing the surface with distilled water. At first glance, someone may think that peak current will be higher for creams (*'more milk fat more vitamins'*) **(Gaucheron, 2011)**. According to Figure 5, demonstrating a dependence of anodic peak current on milk fat content in selected samples, it seems that the assumption is misleading. An explanation could be summarized as follows: lipophilic vitamins present in samples are equally distributed between pasting liquid and milk fat during (*'liquid-liquid'*) extraction and these vitamins are more detained in creams (12 - 31%) than in milks (0.5 - 3.5%) due to many times higher fat content (*'like dissolves like'*).

Extraction repeatability

Assuming that MFGs are homogeneously dispersed throughout the volume of cow's milks and creams and their diameter is not higher than 1 µm **(Robin and Paquin, 1991; Michalski et al., 2004)**, an extraction repeability will be affected only by homogeneity of glassy carbon paste used **(Sýs et al., 2017)**. If recovery of developed HPLC-based methods ranging approximately from 85 to 110% **(Blanco et al., 2000; Gomis et al., 2000)** is taken to account, satisfactory repeatability of extraction characterized by RSD lower than 9% was achieved. Even despite the relatively short error bars (see Figure 5), it is not possible to determine, on the basis of the peak heights received, whether it is cream or milk with a 3.5% fat content.

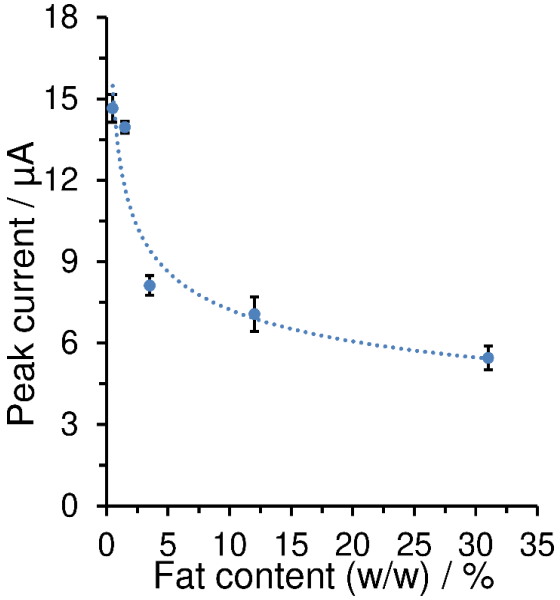

Figure 5 Dependence of peak current response (anodic oxidation of present lipophilic vitamins) on the content of fat in cow's milks and creams.

CONCLUSION

Everyone agrees that the most important part of whole analysis represents a sample preparation at which significant losses of analytes may occur and cannot be detected during the final analysis. Because the extractive stripping voltammetry requires a minimum sample preparation, it was tested as suitable analytical tool for cow's milks and creams quality control. However, the results obtained suggest that direct extraction of lipophilic vitamins (dominantly all-*trans*-retinol) from a continuously stirred sample and subsequent voltammetric detection using square-wave voltammetry could only be used for semi-quantitative determination of milk fat, at this stage of development. Finally, it can be concluded that the scientific hypothesis was refuted.

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Conflict of interest:

All authors declare no conflict of interest.

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EVALUATION OF THE FOLIAR NUTRITION INFLUENCE ON SELECTED QUANTITATIVE AND QUALITATIVE PAPRAMETERS OF SUGAR MAYZE (*ZEA MAYS* SK *SACCHARATA*)

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ABSTRACT
We evaluated the effect of foliar application of fertilizer Tecnokel amino Zn on selected quantitative (weight of one corn cob, average length of corn cob, number of grains per row and number of rows per cob) and qualitative (total yield, total carotenoid content, total sugar content) parameters of sweetcorn. The small trail experiment was founded in 2016 in the Botanical Garden of the Slovak University of Agriculture. We observed 7 selected varieties of sweetcorn and two variants: control and with leaf nutrition. 15 plants we revaluated for each variety under both variants. The corn was grown in three repetitions for each variant. Based on the obtained results, we found that both the qualitative and quantitative parameters were mainly dependent on the genotype. Statistically significant was effect of the variety on the total sugar content in maize grains. Influence of foliar nutrition was not confirmed as statistically significant, but Tecnokel amino Zn has a positive impact on several quantitative parameters as weight of one maize ear or average length of corncobs. Content of total carotenoid doesn´t depend on genotype or variant.

Keywords: sweet corn; carotenoids; total sugars; zinc; foliar application

INTRODUCTION
Sweet corn, also known as green maize or sweet maize in many parts of the world, is a crop of New World origin **(Welbaum, 2015)**. It is one of the oldest crops cultivated on the world. Indigenous people in both Americas have known corn as early as 5000 years ago **(Černý, 2011)**. Now, the USA is a leading producer of sweet corn with 236,860 ha with production totalling 3,788,030 metric tonnes. In Slovakia we have sweet corn production with only 1,110 ha and total yield 7,498 t **(Meravá, 2017)**. Sweet corn (*Zea mays* L var. *saccharata* Strut) is one of several types of maize, which also includes flint corn, dent corn, popcorn, flour corn, and pod-corn but sweet corn differs from other corns. The primary difference is gene expression that determines endosperm carbohydrate content as well as many other genes that affect maize growth. Sweetcorn world consumption has increased over the past 30 years. As consumable part are grains of milk ripeness, while production is focused on three distinct markets: fresh, canning, and freezing **(Alan et al., 2014; Andrejiová and Šlosár, 2015; Khan et al., 2018)**. *Z. mays* var. *saccharata* is a warm-season, frost sensitive, annual monocot which belong to family *Poaceae*. For reaching high yields it´s necessary harmonic and balanced effect of all agroecological factors. Accurate growth and development depend on suitable temperatures and proper water regime in deep humous soils **(Welbaum, 2015)**. In animals, carotenoids are the precursors for vitamin A and serve as important antioxidants to prevent many diseases such as cardiovascular disease, cancer and light-induced erythema. Studies have also shown that dietary intakes of lutein and zeaxanthin can reduce the risks of cataracts and age-related macular degeneration (AMD), which is the leading cause of blindness among the elderly. Corn is one of the essential sources of these carotenoids, contains significant amounts of lutein, zeaxanthin, and other carotenoids. Carotenoid content of sweetcorn can reach up to 1,978 $mg.g^{-1}$ of fresh weight. In recent years, many studies have been carried out on carotenoids in sweetcorn. They observed several parameters, among them changes of carotenoids in sweetcorn during thermal processing or the quantification of carotenoids in different genotypes of sweet corn **(Liu et al., 2017; Scott and Eldridge, 2005)**. Sweetness is the major component of flavour affected by the amounts of sugar and starch in the endosperm. Selection more tender and crispy genotypes with higher sugar, lower starch concentration and more intensive sweet corn aroma would increase the eating quality of the product. But sweetness is determined not only by genetics, but also by the agronomics practices, how the respective varieties are managed and harvested **(Alan et al., 2014)**. Zinc (Zn) is an essential micronutrient for plant life and it is a recommended micronutrient in fertilizer programs for production of corn and sweet corn **(Sutradhar, Kaiser and**

Fernándeza, 2017). To fully explore grain production potential of sweet corn, it is essential to know how plants interact morphologically and physiologically in a community and to realize management practices, which allow them to utilize growth resources in their environment **(Hayat et al., 2018)**.

Scientific hypothesis

Foliar application of Tecnokel Amino Zn had not a significant effect on the qualitative and quantitative parameters of corncob. Used preparation positively affected sugar content and the ear of maize size while carotenoids were not negatively affected. The influence of the variety was statistically significant.

MATERIAL AND METHODOLOGY

The trial establishment

An experiment was founded in 2016 in Botanical Garden of Slovak University of Agriculture (below BG SUA) in the field conditions. Area is situated in very warm agro-climatic region, very dry sub-region. The mean annual temperature was 10 °C (Table 1) with average annual rainfalls 62.4 mm per month (Table 2). Meteorological measurements were carried out by the help of meteorological station in the area of botanical garden, SUA in Nitra. The mean monthly air temperature and average rainfall for the year 2016 were evaluated by the climate normal 1961 – 1990.

In the experiment we investigated the effect of foliar nutrition with Tecnokel Amino Zn on selected quantitative and qualitative parameters in the following variants:

- 1st variant (C) – (control) without applying of Tecnokel Amino Zn.
- 2nd variant (Tecnokel Amino Zn) – foliar application of Tecnokel Amino Zn.

For both variants, based on the agrochemical analysis of the soil and the recommended normatives for the cultivation of sweet corn, we applied nitrogen one week before sowing in the form of nitrogen fertilizer LAD (27% N) (60% of the recommended normatives for corn) at a dose of 72 $kg.ha^{-1}$. When plants were 50 cm tall, we applied 40% of recommended LAD normative (27% N) in dose 48 $kg.ha^{-1}$.

Sowing the seeds was into pre-prepared soil on April 26, 2016. Before sowing, we made soil air spacing and removed the weeds. The seeds were sown in space 0.8 m between rows and 0.3 m within rows. During the vegetation. the supplementary irrigation was applied, as well as loosening and weeding of the crop. As corn tended to expel side shoots, we removed them on 8 June.

In the growth phase of 6 – 8 true leaves, (06.06.2017) we applied foliar fertilizer Tecnokel Amino Zn, which plays a key role as a building material and a regulating factor of wide range of enzymes and positively influences the transport of substances in the plant. The dosage of application was 30 $mL.10L^{-1}$ of water applied to the Tecnokel Amino Zn variant. The second application of Tecnokel Amino Zn with dosage 30 $mL.10L^{-1}$ of water was carried out on 21st July after flowering.

Harvesting of sweet maize ears was carried out gradually according to the maturation of individual varieties, in the period from 26th July to 1st August in the milky matured grains stage. Within each variety we evaluated 15 plants from the control and 15 from Tecnokel Amino Zn variant.

Table 1 Evaluation of the mean monthly air temperature 2 m above ground within the selected months in 2016, according to climatology normal 1961 – 1990.

	t [°C]	Normal 1961-1990	Δt [°C]	Characteristic
V.	15.0	15.1	-0.1	Normal
VI.	20.3	18.0	2.3	Extra Warm
VII.	21.4	19.8	1.6	Warm
VIII.	19.5	19.3	0.2	Normal
IX.	17.5	15.6	1.9	Warm

Table 2 Evaluation of monthly total rainfalls in selected months in 2016, according to climatology normal 1961 – 1990.

	Z [mm]	Normal 1961-1990	% of normal	Characteristic
V.	91	58	157	Extra Wet
VI.	14	66	22	Extremely Dry
VII.	135	52	259	Extremely Wet
VIII.	35	61	57	Dry
IX.	37	40	92	Normal

Table 3 Summary of the observed maize genotypes.

Variety	Supplier	Origin
SF 648 F1	Strube	Spain
Rising sun F1	Strube	Australia
Astronaut	Strube	Australia
ZHY 1312 OR	Strube	Spain
Escalate	Strube	Australia
ZHY 0874 OV	Strube	Spain
Overture	Strube	Australia

Table 4 Agrochemical characteristics of the soil before the foundation of the experiment in 2017.

pH/KCl	humus %	Nutrients content in $mg.kg^{-1}$ of the soil					
		N_{an}	P	K	S	Ca	Mg
7.14	4.17	13.0 M	198.8 VH	487.5 VH	2.5 VH	610 H	816 VH

Note: Nutrient content: M – medium content, H – high content, VH – very high content.

Evaluation of selected quantitative parameters:

In the labs of the Department of Vegetables production at SUA we evaluated the following parameters: weight of one corn cob, length of corn cob, the number of grains per row and the number of rows per cob. Based on the average weight of maize cobs and cultivation spacing, the total yield was calculated.

Laboratory analyses – qualitative parameters

Qualitative characteristics were estimated in the laboratory of Department of Vegetable Production, SUA, in Nitra. For laboratory analysis were used only fresh corn ears in phase of milk grains within of all observed varieties. Weight of one sample – 20g. Both control as well as the Tecnokel Amino Zn variant were observed. Fresh fruit analysis took a place directly after each harvest. Total carotenoids were estimated by spectrophotometric measurement of substances absorbance in petroleum ether extract on spectrophotometer PHARO 100 at 450 nm wavelengths according to **Hegedüsová et al. (2015)**. The determination of total sugars according to **Somogyi (1952)** was carried out at the Department of Agrochemistry and Plant Nutrition.

Statistical analysis

The analysis of variance (ANOVA), the multifactor analysis of variance and the multiple Range test were done using the Statgraphic Centurion XVII (StatPoint Inc. USA).

RESULTS AND DISCUSSION

Total carotenoids

The content of total carotenoids in selected varieties of sweet corn grains without foliar application of Zn ranged from 0.75 to 1.35 mg.100g^{-1} of fresh matter. In the Tecnokel Amino Zn variant, the values ranged from 0.65 to 1.83 mg.100g^{-1}. The highest content of total carotenoids was recorded in the variety Escalante 1.83 mg.100g^{-1} for observed variant. The positive influence of Zn on the content of total carotenoids was observed in varieties Rising Sun, Astronaut, ZHY 1312 OR, Escalate and ZHY 0874 OV (Figure 1). In different experiment **Howe and Tanumihardjo (2013)** reported higher carotenoid content in four varieties of sweet corn ranged from 0.99 to 3.53 mg.100g^{-1}.Based on statistical evaluation of our data obtained by the multifactor analysis of variance, we can state that the variant hadn´t significant influence on the total carotenoids content in fresh corn grains (Figure 4). Effect of the variety on the total carotenoid content wasn´t statistically significant.

Total sugars

Based on the results, we can conclude that all evaluated varieties of sweet corn belong to the Sh-2 group with a storage period of 4 – 7 days. The total sugar content ranged from 3.85% to 8.60% what is comparable with results of **Zaniewicz-Bajkowska et al. (2010)**. They observed the total sugars content in sweet corn in range from 5.61% to 9.02%. In our experiment, the lowest content was recorded for variety ZHY 1312 OR 3.85%. Overture variety obtained highest value of 8.60% (Figure 2). By foliar application of Tecnokel amino Zn at a dose of 30 mL.10L^{-1} of water, we found that the ingested preparation had no statistically significant effect the increase of total sugars in corn grains (Figure 5). Based on statistical analysis, we can conclude that the genotype has a significant effect on the total sugars in the grains at the stage of milk maturity.

Total yield

Haytova (2013) in her review referred that additional foliar application during the growth and development of crops can improve their nutrient balance, which may lead to an increase in yield and quality of crops. Harvesting of maize in our experiment proceeded gradually according to maturation of individual varieties. The average calculated yield achieved in individual varieties and variants is compared in (Figure 3).

During the evaluation of total corn cob yield, we found that within the control variant, total yield ranged from 8.97 t.ha^{-1} in SF 681 variety to 12.52 t.ha^{-1} in Escalate variety. However, the highest hectare yield was 13.14 t.ha^{-1} for the Overture variety without the application of the foliar fertilizer. Similar result was observed also by **Rosa (2015)**, where the average marketable ears yield was 13.0 t.ha^{-1}. The lowest yield per hectare in our experiment reached the SF 681 variety with 8.97 t.ha^{-1}. However, the greatest difference between crops was recorded for the ZHY 1312 variety in favour of the Tecnokel Amino Zn variant 24.01% (Figure 3). Similar results stated also **Fahrurrozi et al. (2016)**. In their study additional foliar fertilization of sweet corn did not significantly influenced growth and yield. **Szczepaniak et al. (2018)** event stated that zinc (Zn) fertilizers applied to maize simultaneously with amino acids (AA) at early stages of its growth may decrease the yield variability due to correcting its nutritional status during the 'critical window' but simultaneously confirm the benefits in order to ameliorate the influence of abiotic stress. However, this combination has positive effect on lettuce plants **(Ghasemi et al., 2013)**. **Grzebisz et al. (2008)** wrote that according to other authors maize plants responded to zinc fertilizer and yielded 10 – 20% more. It supported by positive result of **(Tariq et al., 2014; Liu et al., 2016; Ruffo et al., 2016, Shabaz et al., 2015)**. However, our result may be influenced by an inappropriate proportion of the nutrients applied. **Shabaz et al. (2015)** concluded that this significant enhance of corn yield is because trace elements had a synergic affiliation with other units. **Wang et al. (2017)** and **Iqbal et al. (2016)** also state that Zn combined with K or N can slightly or significantly increased grain yield. The influence of Zn uptake and its effect on corn yield depends on Zn supply in soil **(Eteng et al., 2014)**, what should caused our results. All these results are in consonance with **da Silva et al. (2017)** exhibited increase in corn yield by 0.342 t.ha^{-1} with 83.7% probability of positive response after foliar application of amino acid bio stimulant. **Popko et al. (2018)** confirmed these results. Amino acids bio stimulants increased wheat grain yield and agronomic productivity.

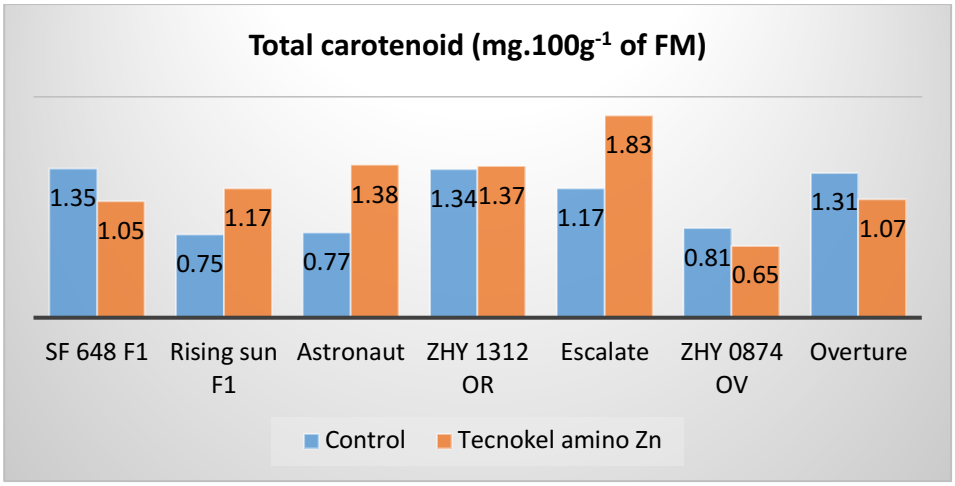

Figure 1 Total carotenoids content (mg.100g^{-1} of fresh matter) in sweet corn grains depending on the observed maize varieties and variants (Nitra, 2017).

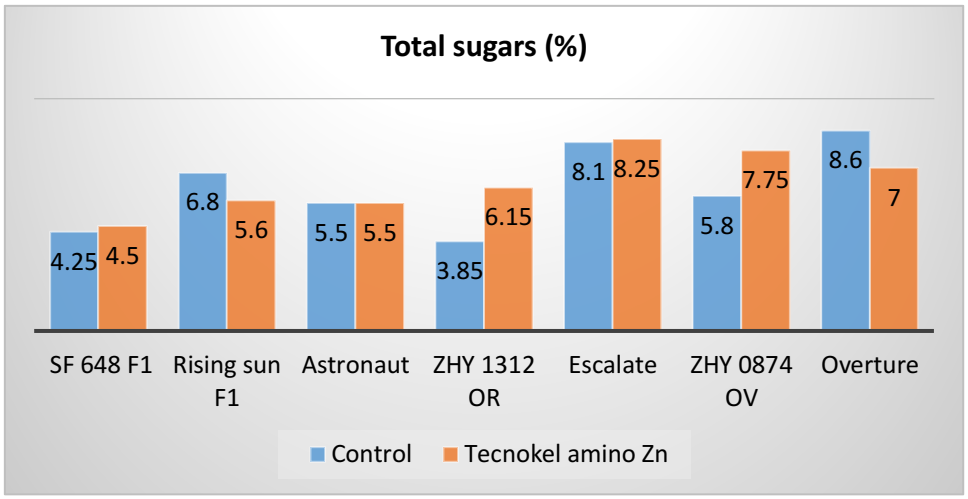

Figure 2 Total sugars content (%) in sweet corn grains depending on the observed maize varieties and variants (Nitra, 2017).

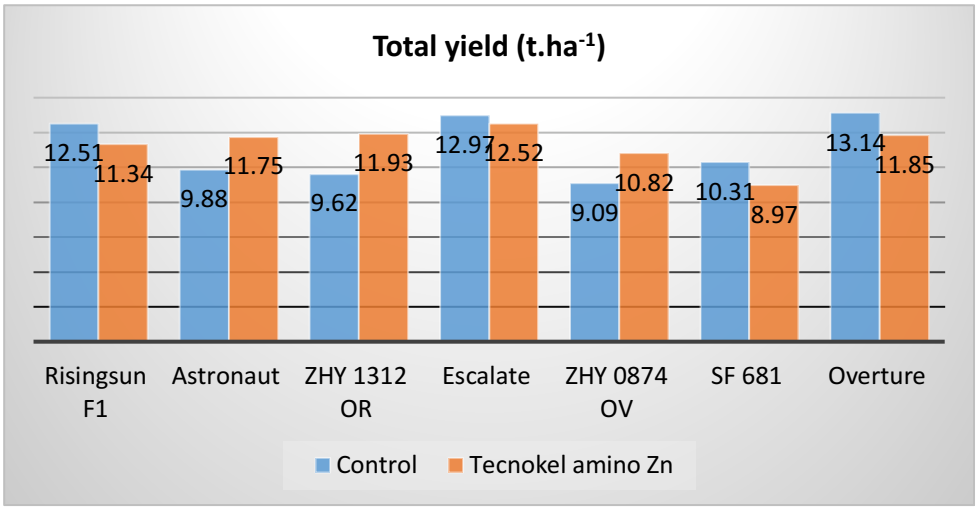

Figure 3 Total yield (t.ha^{-1}) of sweet corn ears depending on the observed maize varieties and variants (Nitra, 2017).

Table 5 Average ear of maize weight (g) depending on the observed maize varieties and variants* (Nitra, 2017).

Variety/variant	C	Tecnokel amino Zn	Difference (%)
Risingsun F1	300.4 ±24.36 c	272.2 ±25.25bc	-9.36
Astronaut	237.3 ±22.81 ab	282.1 ±34.73 cd	+18.87
ZHY 1312 OR	231.1 ±23.82 ab	286.4 ±19.76 cd	+23.92
Escalate	311.4 ±35.38 c	300.6 ±43.12 d	-3.47
ZHY 0874 OV	218.3 ±33.30 a	259.7 ±14.91 b	+18.96
SF 681	247.5 ±16.40 b	215.5 ±20.14 a	-12.93
Overture	315.5 ±30.39 c	284.5 ±26.25 cd	-9.99

Note: *Average ±standard deviation. The different letters of alphabet listed with the mean values in the columns represent statistically significant differences between the observed varieties (p <0.05).

Table 6 Average ears of maize length (cm) depending on the observed maize varieties and variants* (Nitra, 2017).

Variety/variant	C	Tecnokel amino Zn	Difference (%)
Risingsun F1	22.3 ±1.14 c	23 ±0.73 d	+3.13
Astronaut	19.3 ±1.44 ab	19.4 ±0.99 a	+0.51
ZHY 1312 OR	19.9 ±0.54 a	19.4 ±0.75 a	-2.52
Escalate	25 ±0.96 d	24 ±1.14 e	-4.00
ZHY 0874 OV	19.8 ±0.82 b	20.1 ±0.65 ab	+1.51
SF 681	19.9 ±0.54 b	20.2 ±0.86 b	+1.50
Overture	21.9 ±0.59 c	22.3 ±1.23 c	+1.82

Note: *Average ±standard deviation. The different letters of alphabet listed with the mean values in the columns represent statistically significant differences between the observed varieties (p <0.05).

Table 7 Average number of rows in one ear of maize, depending on the observed maize varieties and variants* (Nitra, 2017).

Variety/variant	C	Tecnokel amino Zn	Difference (%)
Risingsun F1	17 ±1.76 a	16 ±1.03 a	+2.58
Astronaut	20 ±0.97 c	20 ±2.15 d	+1.01
ZHY 1312 OR	20 ±1.79 cd	20 ±1.53 cd	-1.99
Escalate	18 ±1.34 b	18 ±1.30 b	+2.25
ZHY 0874 OV	19 ±1.35 c	18 ±1.35 d	-4.24
SF 681	19 ±1.71 c	19 ±1.38 bc	-3.11
Overture	21 ±1.66 d	19 ±1.43 bcd	-8.62

Note: *Average ±standard deviation. The different letters of alphabet listed with the mean values in the columns represent statistically significant differences between the observed varieties (p <0.05).

Table 8 Average number of grains in one rowdepending on the observed maize varieties and variants* (Nitra, 2017).

Variety/variant	C	Tecnokel amino Zn	Difference (%)
Risingsun F1	41 ±2.32c	40 ±2.50 b	-1.47
Astronaut	37 ±1.23 b	38 ±1.72 a	+2.17
ZHY 1312 OR	34 ±2.70 a	38 ±2.12 a	+10.81
Escalate	49 ±1.18 e	48 ±2.40 d	-1.65
ZHY 0874 OV	38 ±1.38 b	41 ±1.24 b	+6.86
SF 681	41 ±1.53 c	40 ±1.68 b	-1.97
Overture	44 ±1.73 d	43 ±2.55 c	-3.41

Note: *Average ±standard deviation. The differnt letters of alphabet listed with the mean values in the columns represent statistically significant differences between the observed varieties (p <0.05).

Foliar supply of Zn in the form of complexed with amino acids resulted in a significant increase of total yield and quality also for other species, for example pistachio nuts **(Najizadeh and Khoshgoftarmanesh, 2018)** or soybean **(Teixeira et al., 2018)**. Using the statistical analysis of variance, statistically significant differences in total yield were not proven between the evaluated varieties or variants.

Ear of maize weight

The weight of the corn cobs is one of the most important features for growers when choosing the right variety of sweet corn. During evaluating the weight of the cobs, we found that the highest average weight reached the variety Overture 315.5 g. The lowest average weight was recorded for variety ZHY 0874 OV 218.3 g (Table 5). In the Tecnokel

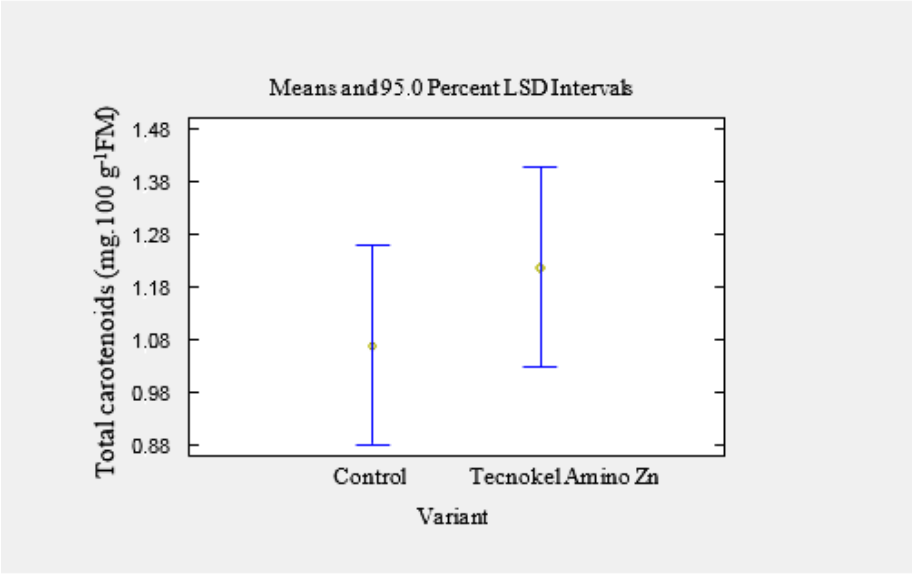

Figure 4 Graphical representation of 95% confidence intervals (p >0.05) for the tested averages of carotenoids content in fresh sweet corn grains and its variants (LSD test).

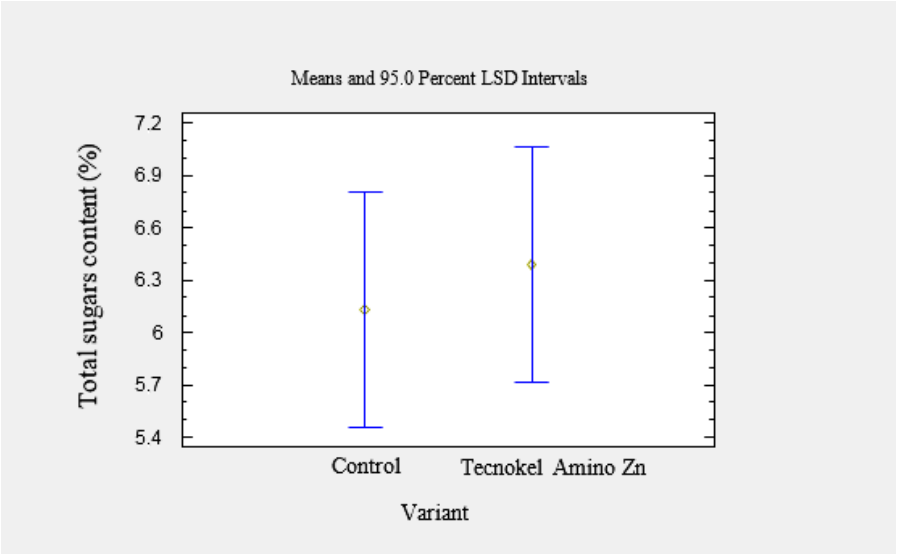

Figure 5 Graphical representation of 95% confidence intervals (p >0.05) for the tested averages of total sugar content in fresh sweet corn grains and its variants (LSD test).

Amino Zn variant, we noted an increase in the weight of the maize-ears for 3 genotypes: Astronaut (by 18.87%), ZHY 0874 OV (by 18.96%) and ZHY 1314 OR where foliar fertilization involved to the highest ears weight grain by 23.92% with average value of 259.7 g. By statistical analysis, we found a statistically demonstrable effect of the variety (genotype) on the weight of the maize ears. We also found that foliar application of Tecnokel Amino Zn fertilizer did not have a statistically significant effect overall, but significantly influenced some individual varieties (Table 5).

Ear of maize length

Barátová (2012) in her work reported results from the evaluation of 8 different varieties of sweet corn. Their ear length ranged from 18 cm to 21 cm. The average length of the strains in our experiment was quite similar and ranged from 19.3 cm to 25 cm. Highest value was caused by extremely long genotype Escalante, which reached average length of corn cobs 25 cm for control and 24 cm for Tecnokel amino Zn variant (Table 6). Due to the foliar application of fertilizer, we observed a slight increase in length for five varieties: Rising sun F1 3.13%, Astronaut 0.51%, ZHY 0874 OV 1.51%, SF 681 1.50%, Overture 1.82%. Statistical evaluation of results showed a statistically significant influence of the variety on ears length (Table 6), but the application of foliar fertilizer Tecnokel Amino Zn did not have a statistically significant effect on the length of corn cobs. It was similar in study **Tadros et al. (2019)** where foliar application of amino acid bio stimulant on sweet corn did not show significant effect on sweet corn ears.

Number of rows in one ear of maize

Strube D&S GmbH gives the following characteristics for cultured genotypes: Rising sun F1 – 14, Astronaut – 20, ZHY 1312 OR – 20, Escalate – 18, ZHY 0874 OV – 18, SF 681 – 20, Overture – 20. For sugar corn, the number of rows

in the ear should be even. In our experiment we recorded average number of rows in the interval between 16 – 21 rows. The highest average value in the number of rows in width 21, was recorded in the Overture variety. By applying zinc to this variety, we have not seen any greater increase in the number of rows. The smallest average values were recorded for the variety Rising sun F1 with 16 rows, but this is more than what the seller report. Also, for variety Overture was measured 8.62% fall in number of rows compared to variant with foliar fertilizer (Table 7). Average values obtained from our tested maize ears were comparable with **Barátová (2012)**. She referred that the average number of rows in ranged with the width of 16 – 18. After statistical analysis of the variance, we found a statistically significant effect of the variety on the number of rows in the ear, but at the same time the effect of the foliar fertilizer Tecnokel Amino Zn was not significant (Table 7).

Number of grains in one row

Number of grains in one row ranged between 34 – 49. The highest average values obtained genotype Escalate with yield 49 grains. The ZHY 1312 OR variety showed the lowest number of grains in row with 34 grains, but for this variety can be declared greatest increase over control variant with 10.81%. In addition to this variety, the number of grains increased in two more cases: ZHY 0874 OV and Astronaut (Table 8). Similar result referred **Mosavifeyzabad et al., (2013)**. They confirmed positive effect of Zn fertilizer on number of corn grains in one row. However, based on statistical analysis of the variance, variety has a significant influence and increase in the number of grains in a row within the variant Tecnokel Amino Zn was not statistically confirmed (Table 8).

CONCLUSION

Foliar application of Tecnokel amino Zn had not a significant effect on the increase of quantitative and qualitative parameters of sweet corn, but positively affected total yield, especially in case of SF 681 and Overture variety as well as some quantitative parameters like average weight of maize ear. This parameter showed better results within 3 varieties with increase about 20%. However, the effect on carotenoids and total sugar was negligible. Based on our results, we can conclude that statistically significant changes were indicated especially between different genotypes. Greatest results reached varieties Escalate and Overture. Variety Overture achieved highest total yield 13.14 $t.ha^{-1}$.

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MINERAL COMPOSITION OF *ALLIUM CEPA* L. LEAVES OF SOUTHERN SUBSPECIES

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ABSTRACT
The mineral composition of *Allium cepa* L. leaves was measured by using the scanning electron microscope of Japanese company JEOL, model JSM600LA with EDS system. 11collection samples of FRC "All-Russian Institute of Plants Genetic Resources named after N. I. Vavilov" and 4 samples of FSBSI "Research Institute of Agriculture of the Crimea" were studied. 12 main elements (in mass., %) contained in onion leaves were evaluated. The samples with the maximum macro- and micronutrient elements accumulation in the leaves used for the sourthern subspecies breeds and hybrids selection were revealed. These samples can be used to prevent the elements deficiency in the human body. The following samples number with a high accumulation of the elements in the leaves was revealed: K – nine (from 20.0 to 3.3 max %: B12132B, trimontzium, Rouge pale, Red Wetherstfield, Blood red flat, Valensiya, Tavricheskiy, Yaltinskiy lux, Yaltinskiy rubin), P – five (from 1.8 to 2.8 mass., %:B12132B, Mestniy, Valensiya, Yaltinskiy lux, Yaltinskiy rubin), Mg – one (2.23 mass., %: Rouge pale), Ca – nine (from 5.4 to 8.3 mass., %: Mestniy, Rouge pale, Mestniy, Red Wetherstfield, Blood red flat, Valensiya, Brown Beauty, Yaltinskiy lux and Yaltinskiy rubin), Fe – two (from 0.5 to 0.8 mass., %: B12132B, Tavricheskiy), S – seven (from 2.2 to 2.5 mass., %: B12132B, Mestniy, Red Wetherstfield, Tavricheskiy, Yaltinskiy lux, Yaltinskiy rubin, Yaltinskiy model No. 3), Na – two (from 1.3 to 1.5 mass., %: B12132B, Mestniy), Cl – five (from 4.0 to 7.0 mass., %: Mestniy, B12132B, Trimontzium, Red Wetherstfield, Yaltinskiy model No. 3), Cu – one (1.9 mass., %: Yaltinskiy model No. 5), Mo – eight (from 5.2 to 7.0 mass., %: Tavricheskiy, Yaltinskiy lux, Yaltinskiy rubin, Yaltinskiy model No. 3, Mestniy, Trimontzium, Red Wetherstfield, and B12132B), Zn – seven (from 0.5 to 4.97 mass., %: Yaltinskii model No. 3, Mestniy, B12132B, Rouge pale, Blood red flat, Brown Beauty, Yaltinskiy rubin) and Si – one (0.5 mass.%,%: Yaltinskiy lux). The order of the elements accumulation variation in the onion samples was distributed as follows: Zn > Fe > Si > Na > P > Cl > Mo > Mg > S > Ca > Cu > K.

Keywords: *Allium cepa* L.; leaves; analytical scanning electron microscopy; energy dispersion X-ray analysis; ash elements

INTRODUCTION
The plants natural feature is their ability to extract and assimilate mineral elements from the soil and water solutions. The following macro nutrient elements are necessary for plants growth and development: N, P, S, K, Ca, Mg, Fe and micro-nutrient elements Cu, Mo, Zn, Mn, B. However, in addition to these elements such useful elements as Na, Cl, Si were also included. They are used in the metabolic processes, with their absence in the environment the plant cannot go through the whole development cycle. The onion green leaves are a source of mineral elements that get into the human body in the form of ions in balanced concentrations. The soil and other multifactorial conditions that accompany the onions growth affect the onions mineral composition **(Golubkina, Agafonov and Dudchenko, 2009; Golubkina et al., 2015)**.

Onions - the main vegetable crop, it is actively used in the food and canning industry, modern medicine. Onions are consumed in the fresh, fried, boiled form, used for salads, minced meat, in the vegetables canning, in the meat and fish industry **(Borisenkova, 1993; Vodyanova and Alpysbaeva, 2004)**. Green onions and garlic are recommended for eating during flu epidemics, at atherosclerosis and heart disease, especially if the basis of nutrition is foods high in fat and low in fiber. Onions in this case suppress the cholesterol synthesis, reduce the level of fibrinogen (complex protein, blood plasma glycoprotein, the most important blood clotting ability component) **(Galkin et al., 2000)**. Onions are potential sources of prostaglandins, substances that regulate blood pressure **(Platonova, 2000; Agafonov et al., 2005)**. An important component of the onion chemical composition is quercetine, used in combination with vitamin C as a vasodilator **(Danikov, 1998; Ulyanova, 1998; Platonova, 2000)**.

The biochemical composition of both bulbs and green leaves varies in different development stages depending on

the breed, environmental and agro-technical conditions of the plant growing **(Ananyina, and Glukhova, 1988; Dudchenko, 2009; Kielak et al., 2006; Nemtinov et al., 2019)**.

The following onions nutritional value is given by **Skurikhin and Tutelyan (2007)**, mg per 100 g product: K – 175, Ca – 31, Mg – 14, Na – 4, S – 65, P – 58, Cl – 25, Fe – 0.8, Cu – 0.09, Zn – 0.85 mg and other trace elements. The elements content of K – 259, Ca – 100, Mg – 18, Fe – 1.0 ($mg.100g^{-1}$) in green onions significantly exceeds the analogue parameters of onion bulbs, but the lower level of Zn – 0.39 $mg.100g^{-1}$. The development of the latest technologies in physiological and medical research confirms the important role of micro nutrient elements in metabolic reactions and submolecular processes the activity of which depends on the presence of certain macro- and micro nutrient elements in the daily human diet **(Avtsyn et al., 1991; Kavita and Puneet, 2017; Motyleva et al., 2017)**. The purpose of our work is to study the peculiarities of mineral elements accumulation in 15 samples of *Allium cepa* L., including 11 collection ones, originating from 9 countries of the world and 4 ones selected by FSBSI "RIA of the Crimea".

Scientific hypothesis

There are no comparative data on the mineral composition of the different samples of *Allium cepa* L. onion leaves, originating from 9 countries of the world, grown in the conditions of the Crimea. We evaluated differences in macro- and micronutrient content in the range of consumer properties of hub varieties.

MATERIAL AND METHODOLOGY

The objects under study were *Allium cepa* L. collection samples. The onions collectable and commercial material was grown in FSBSI "RIA of the Crimea" fields. The green onions leaves were prepared for the mineralization at the bulbs early formation stage (Table 1).

The content of each element in the onion samples was conventionally divided into the groups: high, medium, low.

The soils of FSBSI "RIA of the Crimea" experimental and production base are represented by the southern carbonate black earth. The onion plants were grown on the pure (background) soils uncontaminated with high-density metals (within permissible rates accepted in Russia).

The data of the quantitative elemental composition, given in the present paper, are taken in the laboratory of physiology and biochemistry of the Centre of the plants genofond and bioresources of Federal State Budgetary Scientific Institution All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery, Moscow. The researches are original and are fulfilled with the usage of the modern analytical equipment. The average seeds weighing with the mass of 10 g was mineralized in the muffle furnace Naberterm (Germany) at T = 400 ºC. The received ash was dispergated by ultrasound at 18 kHz frequency for 15 minutes. The dispergate even layer was applied on the object table covered with carbonic scotch.

The chemical composition of the basic ash components (Na, P, S, K, Mn, Fe, Mg, Ca, Al, Si, Cl, Zn, Se, Mo) was determined by the method of energy dispersive spectrometry (ESD) on the analytical raster electron microscope JEOL JSM 6090 LA. The microscope resolution is 4 nm at accelerating voltage 20 kV (secondary electrons image), zooming is from x 10 till x 10 000. While performing the elemental analysis the working distance (WD) is 10mm. Energy-dispersive spectrometer allows to carry out the quantitative X-ray microanalysis with the desired analysing area: in a point or a really, and to receive the maps of elements allocation. X-ray microanalysis data are presented in the form of standard protocols which contain the microstructure picture of the sample under study, the table of the data in weighting and atomic correlation, spectra and histograms. The spectrum example is shown in Figure 1.

Statisical analysis

Taking into consideration the spectrum lines intensity the concentration of the desired element can be determined. The fractional accuracy of the chemical analysis is spread in the following way: at the element concentration from 1 till 5% the accuracy is less than 10%; from 5 till 10% the accuracy is less than 5%; at the element concentration more than 10% the accuracy is less than 2%. 100 ash areas of each sample were studied. The local analysis is 3 mm, the scanned area is not less than 12 μm. We used the statistical analysis of the Excel package (Microsoft Excel, v. 2016).

Table 1 *Allium cepa* L. onion samples.

Sample name	Origin
Collectable samples	
Mestniy	Russia
B12132B	Australia
Southport red	the USA
Trimontzium	Bulgaria
Tavricheskiy	Russia
Rouge pale	Algeria
Mestniy	Azerbaijan
Red Wetherstfield	Bolivia
Blood red flat	the Netherlands
Valensiya	Portugal
Brown Beauty	the USA
Samples selected by FSBSI "RIA of the Crimea"	
Yaltinskiy model No. 3	Russia
Yaltinskiy model No. 5	Russia
Yaltinskiy lux	Russia
Yaltinskiy rubin	Russia

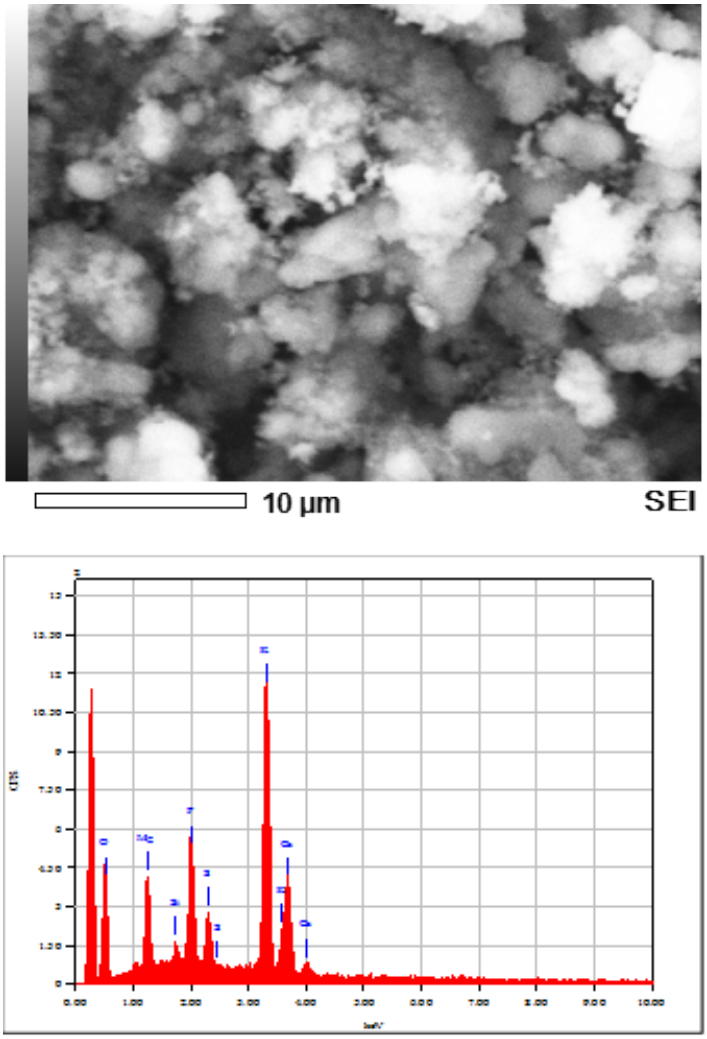

Figure 1 The microstructure picture of the sample under study (1) and the general view of the X-ray spectrum lines that show the elements presence in the analyzing area (2).

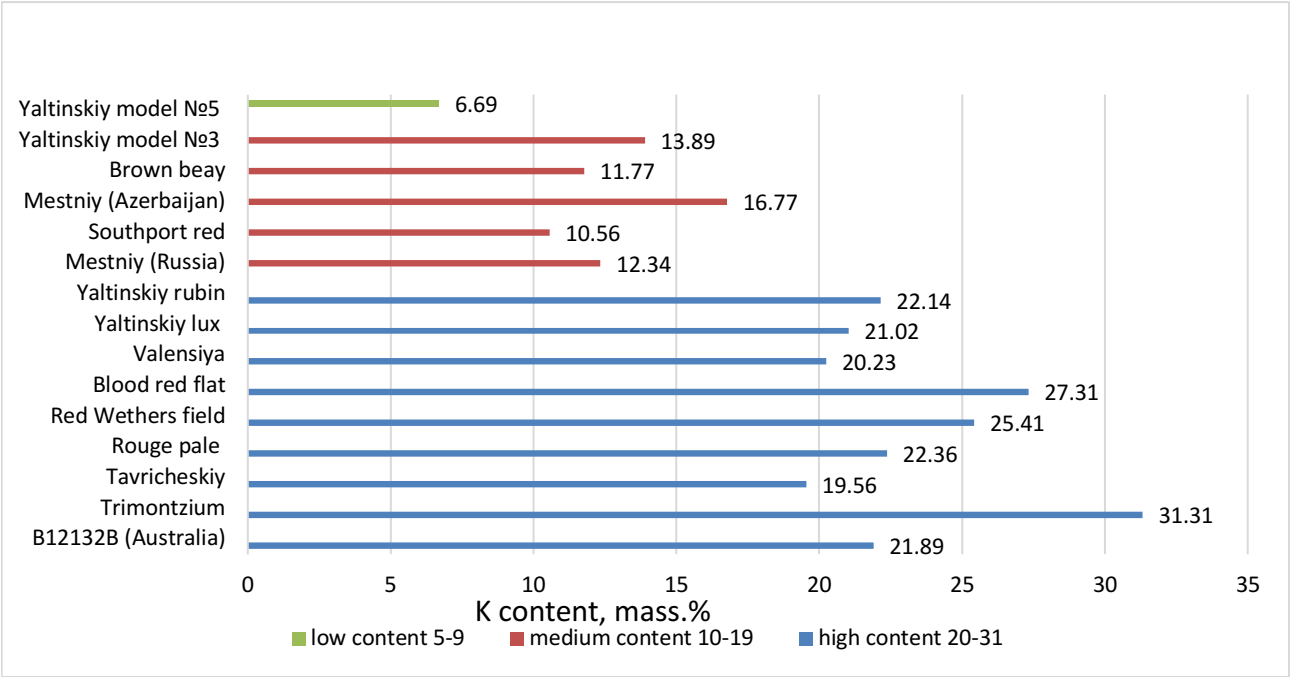

Figure 2 The cultivar differences at K accumulation in *Allium cepa* L. leaves.

RESULTS AND DISCUSSION

Onions or young leaves are used for eating in onion crops. Green leaves have less dry solids and soluble carbohydrates than bulbs, but contain a significant amount of nitrogen compounds, minerals and vitamins. While having hepatic and bile ducts diseases, traditional medicine recommends eating 100 grams of green onion daily, as it contains a significant amount of trace elements the daily dose of which for a person is 200 mg. However, the role of micro-nutrient elements is not yet fully investigated, new data that constantly appear contradict the previous ones. In this regard, we analysed 12 most significant macro and micro-nutrient elements found in green onion leaves. 17 trace elements were found in the onion's ash. Micro-nutrient elements – Cu, Zn, Co, Mo are parts of enzymes and participate in their activation, improve the plants growth and development.

K is necessary for the muscle contractions, the heart muscle normalization, the nerve cells activity, the blood osmotic concentration, the acid-alkaline and water balance. It controls the transmembrane potential of osmotic pressure, the cathode-anion balance, the pH of cell hemostasis. In ion form K increases the concentration of other ions and is found in all the human body organs **(Meathnis et al., 1997)**.

The highest value of K from 20 to 31 mass., % was accumulated in 9 samples of *Allium cepa* L. onion leaves originating from 7 countries: B12132B (Australia), Trimontzium (Bulgaria), Tavricheskiy (Russia), Rouge Pale (Algeria), Red Wetherstfield (Bolivia), Blood red flat (the Netherlands), Valensiya (Portugal), 2 breeds selected by FSBSI "RIA of the Crimea" - Yaltinskiy lux and Yaltinskiy rubin (Figure 2). The other 5 samples from different places of origin contained a medium amount of K in the leaves - from 10.6 to 16.8 mass., % and the sample Yaltinskiy model No. 3 (Russia) accumulated only 6.7 mass., % of K.

In the paired correlation ratios of K the medium connection was revealed with Mg, Cl, S, Cu at r = 0.4 – 0.6 and the high correlation with Mo at r = 0.7 and the low one with Na, Ca and Zn at r = 0.16 – 0.24. The connection with Fe was practically absent (r = 0.02) (Figure 3).

P (phosphorus) is a part of humans and animals skeleton, more than 50% of it is represented in tissues in the form of inorganic compounds. It is an integral part of DNA, RNA, phospholipids, ATF, ADP, where it performs a structural function. Its role in cellular energy is great.

Its greatest value in the onion leaves ash is noted in 6 samples: Blood red flat (the Netherlands), B12132B (Australia), Mestniy (Azerbaijan), Valensiya (Portugal), Yaltinskiy lux and Yaltinskiy rubin (Russia). The medium rates (1.2 – 1.4 mass., %) are found in 3 samples: Trimontzium (Bulgaria), Yaltinskiy model No. 3 (the Crimea) and Rouge pale (Algeria). The low level of P content (0.4 – 0.7 mass., %) is marked in 5 samples: Mestniy (Krasnodar, Russia), Southport red (the USA), Red Wethers field (Bolivia), Brown beauty (the USA), Yaltinskiy model No. 5 (Russia). And a very low value (0.22 mass., %) is identified in Tavricheskiy (Russia).

In the paired correlation ratios of P a medium connection was revealed with S, K, Fe and Mo at r = 0.3 – 0.6 and an insignificant correlation with Ca at r = 0.2 and a very low one with Cl, Cu and Zn at r = 0.01 – 0.02 (Figure 3).

Mg (magnesium) is necessary for Ca absorption, the metabolism of glucose, amino acids, fats, the nutrients transportation, is involved in the process of protein synthesis, the nerve signals transmission. It is necessary for the cells, tissues and organs regeneration processes. It activates a large number of enzymes that are involved in CO_2 and N consumption process. It is necessary for keeping the cathode-anion and pH balance.

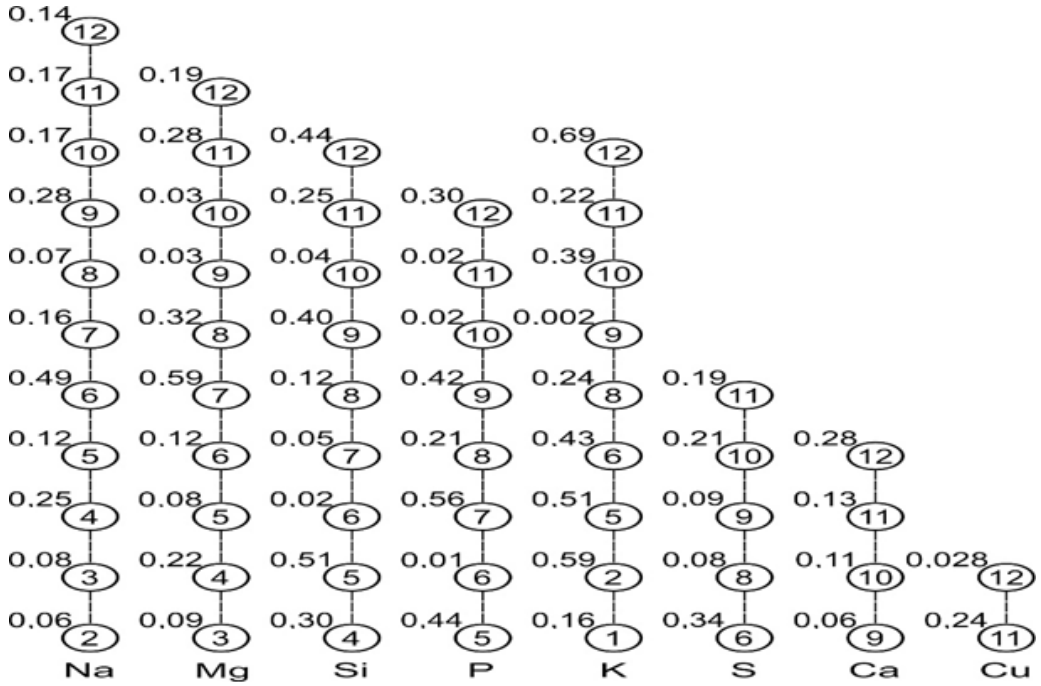

Figure 3 The paired values of the elements content correlation coefficients in *Allium cepa* L. onion leaves: 1 – Na, 2 – Mg, 3 – Si, 4 – P, 5 – S, 6 – Cl, 7 – K, 8 – Ca, 9 – Fe, 10 – Cu, 11 – Zn, 12 – Mo.

Its highest value was found only in one onion leaves sample (2.23 mass.,%): Rouge pale (Algeria), in the other 6 samples the medium rates are marked: 1.1 – 1.5 mass., %: B12132B (Australia), Southport red (the USA), Trimontzium (Bulgaria), Red Wetherstfield (Bolivia), Valensiya (Portugal).

Mg low value (0.4 – 1.0) was presented in 9 samples: Mestniy (Russia), Tavricheskiy (Russia), Mestniy (Azerbaijan), Blood red flat (the Netherlands), Brown Beauty (the USA) and 4 samples selected by FSBSI "RIA of the Crimea": Yaltinskiy model No. 3, Yaltinskiy model No. 5, Yaltinskiy lux and Yaltinskiy rubin.

In the paired correlation ratios of Mg the medium connection was revealed with K, Ca and Zn at r = 0.3 – 0.6, an insignificant correlation with Si, P, S, Cl, Fe and Cu at r = 0.08 – 0.22 (Figure 3).

Ca (calcium). The major part of the human skeleton and teeth consists of Ca. Its ions are involved in blood clotting processes, as well as in providing constant osmotic pressure. It is involved in the cell growth and development processes, is a part of enzymes, affects the metabolism and immunity **(Gusev, 1998; Gins and Gins, 2011)**.

In the onion leaves ash Ca is represented at high rates (5.43 – 8.27 mass., %) in 9 samples: Mestniy (Russia), Rouge pale (Algeria), Mestniy (Azerbaijan), Red Wetherstfield (Bolivia), Blood red flat (the Netherlands), Valensiya (Portugal), Brown Beauty (the USA) and 2 breeds selected by FSBSI "RIA of the Crimea" - Yaltinskiy lux and Yaltinskiy rubin.

Five sort samples showed a medium content (3.74 – 4.92 mass., %) – B12132B (Australia), Southport red (USA), Trimontzium (Bulgaria), Tavricheskiy (Russia), Yaltinskiy model No. 3 (Russia) and a low level one (1.93 mass., %) – Yaltinskiy model No5 (Russia).

In the paired correlation ratios of Ca there was revealed the correlation with Mo at r = 0.3, with Fe, Cu and Zn at r = 0.06 – 0.13 (Figure 3).

Fe (iron) is the main active element of blood hemoglobin, is a part of other cells enzymes, it catalyzes the breathing processes in them. Organic Fe is an important compound of the body, it is a part of many oxidation-reduction enzymes.

In the onion ash 2 samples had an increased Fe accumulation (0.49 – 0.78 mass., %): B12132B (Australia) and Tavricheskiy (the Crimea). The other 9 samples had the medium Fe accumulation (0.11 – 0.21 mass., %): Mestniy (Russia), Trimontzium (Bulgaria), Rouge pale (Algeria), Blood red flat (the Netherlands), Red Wetherstfield (Bolivia), Valensiya (Portugal), Brown Beauty (the USA) and 2 breeds from the Crimea - Yaltinskiy lux and Yaltinskiy rubin. The low Fe accumulation (0.06 – 0.08 mass., %) was found in 4 samples: B12132B (Australia), Mestniy (Azerbaijan) and Yaltinskiy model No. 3 and Yaltinskiy model No. 5 (Russia).

In living organisms S (sulfur) regulates the synthesis of the protein quantity and quality, is a part of its composition, has antioxidant activity. It ensures the process of the electrons energy transfer in the cell. It participates in the methyl groups transportation and fixation, in the production of various hydrogen compounds, which allows the transmission of genetic information **(Gins et al., 2018)**.

The highest S accumulation (from 2.16 – 2.5 mass., %) was found in 7 onion samples: B12132B (Australia), Tavricheskiy (Russia), Mestniy (Azerbaijan), Red Wetherstfield (Bolivia), Yaltinskiy model No. 3, Yaltinskiy lux and Yaltinskiy rubin (Russia). The medium content (1.34 – 1.69 mass., %) was noted in 4 samples: Trimontzium (Bulgaria), Rouge pale (Algeria), Blood red flat (the Netherlands), Valensiya (Portugal). The other 4 samples were characterized by the low S accumulation (0.6 – 0.8 mass., %) in the onion leaves ash: Mestniy (Russia), Brown Beauty (the USA), Southport red (the USA) and Yaltinskiy model No. 5 (Russia).

In the paired correlation of S the medium connection was marked with Cl at r = 0.3, with other macro- and micro nutrient elements (Ca, Fe, Cu and Zn) the low one at r <0.3 (0.08 – 0.21) (Figure 3).

Na (sodium) a part of the plants enchylema that creates high osmotic pressure, is found mainly in intercellular fluid. Na in combination with K is involved in the membrane potential creation, enzymes and muscle contractions, acid-alkaline and water balance activation provides membrane transfer **(Avtsyn et al., 1991)**. A great number of Na salts are found in green onions, garlic, beetroot, parsley, tomatoes and dill.

Na accumulation in the samples of *Allium cepa* L. onion leaves was marked in the following content (mass.,%): high (1.28 – 1.47) Mestniy (Russia), B12132B (Australia); medium (0.5 – 0.9) Trimontzium (Bulgaria), Rouge pale (Algeria), Mestniy (Azerbaijan), Red Wetherstfield (Bolivia), Blood red flat (the Netherlands), Brown Beauty (the USA); the other 7 samples showed low and very low values.

In the paired correlation ratios of Na, the medium connection was revealed with *Cl* and Fe at r = 0.49 and 0.28, with other elements the connection was low at r = 0.12 – 0.25 and very low at r = 0.06 – 0.08 (Figure 3).

Cl (chlorine) is one of the biogenic elements, a constant component of plants, human and animals' tissues. Cl-ions together with Na and K atoms are involved in osmotic equilibrium maintenance and acid-alkaline equilibrium regulating. Na chloride plays a major role in chemical composition homeostasis and water-salt exchange maintenance helping to keep water in tissues. Cl is also an integral part of hydrochloric acid found in gastric juice and actively affects digestion.

Cl accumulation of in the samples of *Allium cepa* L. onion leaves was marked in the following content (mass.,%): high (4.25 – 6.88) Mestniy (Russia), B12132B (Australia), Trimontzium (Bulgaria), Red Wetherstfield (Bolivia) and Yaltinskiy model No. 3 (Russia), medium (3.44 – 3.69) Southport red (the USA), Tavricheskiy (the Crimea) and Valensiya (Portugal); the other 5 samples contained low rates: low 1.99 – 2.99 and one sample very low 0.79.

Cu (copper) is an important essential micronutrient element in human metabolism, as it is connected with enzymes, hormones and vitamins **(Fraga, 2005)**. According to the U.S. Institute of Medicine and the European Union's Food Science Committee, the daily requirement of an adult is 1 – 1.5 mg. Cu carries out the biological mechanism of enzyme biocatalysis, electron transfer, interaction with Fe. It participates in the generational organ and hemoglobin formation, growth and development processes, is a part of melanin, i.e. together with Fe, Mn, Zn, Cu, Se Cu belongs to the 4th group of Mendeleev Periodic System and is an essential micro nutrient element for humans and mammals **(Avtsyn et al., 1991)**. The importance of onion

Table 2 The cultivar diffrences at Cu, Mo, Zn, Si accumulation in *Allium cepa* L. leaves.

The sample name, (origin)	Micronutrient elements (mass %, ±*SD*)			
	Cu	Mo	Zn	Si
Mestniy (Krasnodar, Russia)	1.11 ±0.14	1.86 ±0.13	0.49 ±0.05	0.06 ±0.01
B12132B (Australia)	1.09 ±0.06	6.14 ±0.04	0.58 ±0.01	0.24 ±0.04
Southport red (the USA)	0.88 ±0.07	1.73 ±0.03	0.28 ±0.01	0.03 ±0.01
Trimontzium (Bulgaria)	1.06 ±0.11	5.52 ±0.44	0.22 ±0.01	0.14 ±0.02
Tavricheskiy (the Crimea)	1.23 ±0.11	6.83 ±0.31	0.11 ±0.02	0.07 ±0.01
Rouge pale (Algeria)	0.69 ±0.01	3.54 ±0.22	0.65 ±0.02	0.07 ±0.01
Mestniy (Azerbaijan)	1.11 ±0.11	5.49 ±0.24	0.22 ±0.02	0.29 ±0.02
Red Wetherstfield (Bolivia)	0.12 ±0.01	5.59 ±0.33	0.2 ±0.01	0.12 ±0.01
Blood red flat (the Netherlands)	0.88 ±0.01	2.68 ±0.24	0.47 ±0.04	0.08 ±0.01
Valensiya (Portugal)	1.41 ±0.13	4.01 ±0.14	0.07 ±0.01	0.13 ±0.01
Brown Beauty (the USA)	0.89 ±0.02	1.35 ±0.11	0.68 ±0.01	0.09 ±0.02
Yaltinskiy model No. 3 (the Crimea)	0.77 ±0.01	5.15 ±0.25	4.97 ±0.15	0.29 ±0.01
Yaltinskiy model No. 5 (the Crimea)	1.93 ±0.14	0.44 ±0.01	0.33 ±0.01	0.15 ±0.06
Yaltinskiy lux (the Crimea)	1.15 ±0.09	5.88 ±0.54	0.31 ±0.01	0.51 ±0.05
Yaltinskiy rubin (the Crimea)	1.32 ±0.10	5.42 ±0.45	0.82 ±0.03	0.06 ±0.02

Note: Means within a column with at least one identical superscript are not significantly different by Student`s t-test (p <0.05).

consumption as a source of Fe, Mn and Cu for humans is great **(Golubkina et al., 2013; Skalnaya et al., 2004)**.

According to Cu accumulation the highest value is noted in 1 sample (1.93 mass.,%) Yaltinskiy model No. 5 (Russia) (Table 2), medium one was found in 8 samples (1.0 – 1.5 mass., %): Mestniy (Russia), B12132B (Australia), Tavricheskiy (Russia), Mestniy (Azerbaijan), Valensiya (Portugal) and breeds Yaltinskiy lux and Yaltinskiy rubin (Russia). Low Cu accumulation had 6 samples (0.12 – 0.9 mass., %): Southport red (the USA), Rouge pale (Algeria), Red Wetherstfield (Bolivia), Blood red flat (the Netherlands), Brown Beauty (the USA) and Yaltinskiy model No. 3 (Russia).

In the paired correlation ratios of Cu low connection was revealed with Zn at r = 0.24 and a very low correlation with Mo at r = 0.028 (Figure 3).

Mo (molybdenus) ensures the enzyme catalysis mechanisms, as well as the electrons transfer, is involved in the aminoacids synthesis, in the vitamins C, E, and B12 exchange **Avtsyn et.al. (1991)**. A human's daily need for Mo is 0.5 mg. Mo accumulates mainly in the liver, the kidneys, the internal secretion glands and the skin.

The high Mo accumulation was found in 8 samples: Yaltinskiy model No. 3, Yaltinskiy rubin (the Crimea), Mestniy (Azerbaijan), Trimontzium (Bulgaria), Red Wetherstfield (Bolivia), Yaltinskiy lux (the Crimea) and B12132B (Australia) (from 5.15 – 6.14 mass., %) with the highest value in Tavricheskiy (the Crimea) (6.83 mass., %) (Table 2). The medium Mo content was revealed in 3 samples (from 2.68 – 4.01 mass., %): Blood red flat (the Netherlands), Rouge pale (Algeria) and Valensiya (Portugal); the low rates – in 3 samples (from 1.35 – 1.86 mass., %): Southport red (the USA), Brown Beauty (the USA), Mestniy (Russia) and Yaltinskiy model No. 5 (Russia) showed the lowest value (0.44 mass., %).

Zn (zinc) stabilizes the molecules structure, plays an important role in DNA and RNA metabolism, in the protein synthesis and cells division, in the process of signal transmission inside the cell **(Nechaev, Trauberg and Kochetkova, 2007)**. There are plants-concentrators and even superconcentrators, which accumulate micronutrient elements and can be used for Zn deficiency treatment and prevention measures in the human body.

In our example, *Allium cepa* L. samples accumulated a very high content (4.97 mass.,%) in Yaltinskiy model No. 3 (Russia) and the high one in 6 samples (0.47 – 0.82 mass., %): Mestniy (Russia), B12132B (Australia), Rouge pale (Algeria), Blood red flat (the Netherlands), Brown Beauty (the USA), Yaltinskiy rubin (Russia) (Table 2). The other 7 samples accumulated Zn at low values (0.2 – 0.33 mass., %), with the highest rate in Yaltinskiy model No. 5 (Russia). A very low *Zn* content (0.07 – 0.11 mass., %) corresponded to two breeds - Tavricheskiy (Russia) and Valensiya (Portugal).

Si (silicon) is an obligatory element for plants **(Kolesnikov and Gins, 2001)**. It is accumulated in large quantities in leaves, especially in the leaves and roots conductive tissues. Si is not only the basis of tissues, but it also controls a number of biological and chemical processes in humans and animals. The skin, tendons, vascular walls elasticity is due mainly to *Si* contained in them. *Si* increases the plants specific resistance to abiogenic stresses.

Si accumulation in the samples of *Allium cepa* L. onion leaves was noted in the following rates, mass., %: high (0.5) Yaltinskiy lux (Russia), medium (0.21 – 0.3): B12132B (Australia), Mestniy (Azerbaijan) and Yaltinskiy model No. 3. (Russia), low (0.11 – 0.2): Mestniy (Russia), Trimontzium (Bulgaria), Red Wetherstfield (Bolivia) and Yaltinskiy model No. 3 (Russia) and a very low (0.03 – 0.1): (6 samples) (Table 2).

Cu and Zn content in plant products controlled by sanitary standards, which are 5 $mg.kg^{-1}$ (Cu) and 10 $mg.kg^{-1}$ (Zn) **(SANPIN 2.3.2 1078-01, 2013)**. According to our data, the content of Cu and Zn in the studied onion samples does not exceed permissible norms.

CONCLUSION

Allium cepa L. onion samples, the concentrators, with the highest accumulation of macro- and micronutrient elements in the leaves have been studied. The breeds that can be used for the selection purposes and elements deficiency prevention in the human body were revealed. The following number of the samples with high elements accumulation in the leaves were identified: K – nine, P – five, Mg – one, Ca – nine, Fe – two, S – seven, Na – two, Cl – five, Cu – one, Mo – eight, Zn – seven and Si – one.

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MILK YIELD AND SOMATIC CELLS IN DAIRY EWES WITH RESPECT TO THEIR MUTUAL RELATIONS

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ABSTRACT

The objective of this study was to analyze milk yield and somatic cell count (SCC) expressed as somatic cell sore (SCS) in Lacaune dairy breed. Data from milk performance testing recorded between 2016 and 2018 (farm in West Slovakia) were used. A total, 377 individual milk yield and SCC records of 61 ewes (first, second and third lacation, respectively) were analysed. Mixed model for milk yield included fixed factors: SCC class (lowest, low, middle, high and highest), year of measurement, lactation number, month in milk and interaction between month in milk and SCC class, and random factors of ewe and error. Mixed model for SCS included milk yield class (lowest, low, middle, high, highest), year of measurement, lactation number, month in milk and interaction between month in milk and milk yield class. Random factors of ewe and error were considered as well. Milk yield was significantly affected (p <0.05 or p <0.01) by all investigated factors. Except for interaction between month in milk and milk yield class, the remaining factors significantly affected (p <0.05 or p <0.01) also SCS. The analyses confirmed that SCC may be used as a useful indicator of udder health. It may help in identifying infected ewes, and thus, avoiding mammary infections to be spread throughout the whole flock.

Keywords: Lacaune; milk yield; somatic cell count and score; SCC; Slovakia

INTRODUCTION

Dairy sheep sector is a traditional branch of livestock in Slovakia. In order to be competitive, an increase of milk yield of good quality remains one of the most important goals od sheep farms. However, this aim may be a potential risk for udder health. Consumers, on the other hand, are more iterested in welfare of animals **(Tančin et al., 2019)**, when deciding which food to buy. Types of breeding systems and (also welfare) thus influence both ewe production abilities and health/desease conditions. Somatic cells are considered to be of a negative effect on health of mammary gland and are used for detection of udder infection in ewes **(Gonzalo et al., 1994; Gonzáles-Rodríguez et al., 1995; Tvarožková et al., 2019)**. The consequence of increased SCC is decreasing raw milk quality, which has further consequences for milk processing **(Hag, 2001)**. Mastitis is a costly health problem in dairy ewes **(Arias et al., 2012)**; mammary infections damage udder tissue **(Burriel, 1997)**.

Tvarožková et al. (2019) summarised knowledge about defining the physiological/pathological levels of somatic cell count (SCC) and of proposing the possible tresholds for healthy mammary gland in ewes **(Pengov, 2001; Berthelot et al., 2006; Sutera et al., 2018)**. These values vary among dairy sheep breeds and no single value to differentate between uninfected and infected udder was accepted **(Berthelot et al., 2006; Tančin et al., 2017)**. For example, tresholds for healthy udders in ewes may be as follows: $265x10^3$ somatic cells.mL^{-1} **(Caboni et al., 2017)**, $300x10^3$ somatic cells.mL^{-1} **(Kern et al., 2013)** or $500x10^3$ somatic cells.mL^{-1} **(Sutera et al., 2018)**. **Gonzalo et al. (1994)** and **El-Saied, Carriedo and San Primitivo (1998)** recommended SCC values ranging from 2.5×10^5 to 3×10^5 cells.ml^{-1} as thresholds between healthy and infected udders. According to **Jaeggi et al. (2003)**, thresholds above $1000x10^3$ somatic cells.mL^{-1} decrease the cheese yield and increase the development of rancid flavours in the cheese.

No routine determination of SCC in individual ewes is undertaken on national level in Slovakia; however, there are farms interested in SCC to be known due to fact that costs to cure infected individuals and the decrease of milk yield may affect the profitability. In Slovakia, reports aimed at investigation of SCC and distributions of ewes in respective SCC classes as well as their influence on milk yield and composition were published **(Idriss et al., 2015; Tančin et al., 2017)**; possible values that enable to distinguish between ewes infected/uninfected with mastitis were discussed **(Oravcová, Mačuhová and Tančin, 2018; Tvarožková et al., 2019)**.

In spite of fact that some analyses were done, this study was aimed at providing in-depth investigation of mutual relations between SCC and milk yield on a level of a

single farm. Purebred Lacaune ewes were included in the analysis.

The hypothesis was as follows: SCC negatively influences amount of milk yield; vice versa amount of milk yield negatively influence SCC.

MATERIAL AND METHODOLOGY

Data were collected from the farm located in western Slovakia during the period of three years (from 2016 to 2018). Milk yield and somatic cell count (SCC) of Lacaune (LC) ewes were analysed. Test-day records were taken once per month (under the the guidance of certificated organisation for milk recording i.e. Plemenárske služby, š. p. SR Bratislava). Ewes were machine milked two times per day after lambs were weaned. However, only morning milkings were taken into account.

A total of 667 records of 61 ewes with 95 lactations i.e. 1.56 lactation per ewe) were included. Ewes were in their first, second and third lactation, respectively.

Ewes predominantly lambed in February and March. According to their lambing, ewes were on their second to sixth month in milk (MIM): MIM 2 (30 to 60 days after lambing), MIM 3 (61 to 90 days after lambing), MIM 4 (91 to 120 days after lambing), MIM 5 (121 to 150 days after lambing) and MIM 6 (151 and 180 days after lambing). Due to only six measurements taken between 181 and 194 days, these were included in MIM6. At least, ewes with three test-day records per lactation were considered.

According to SCC, five classes were formed: lowest SCC (under or equal to 200×10^3 cells.mL^{-1}), low SCC (between 200×10^3 and 400×10^3 cells.mL^{-1}) middle SCC (between 400×10^3 and 600×10^3 cells.mL^{-1}), high SCC between 600×10^3 and 1000×10^3 cells.mL^{-1}) and highest SCC (above 1000×10^3 cells.mL^{-1}). Because of non-normal distribution of SCC, these values were transformed and somatic cell score i.e. SCS= $\log_2$(SCC/100000)+3, as mentioned by **Riggio et al. (2007)**, was analysed. According to milk yield (MY), five classes were also formed: lowest MY (under or equal to 200 ml), low MY (between 200 and 400 ml) middle class MY (between 400 and 600 ml), high MY between 600 and 1000 ml) and highest MY (above 1000 ml).

The mixed model methodology using MIXED procedure **(SAS 9.2, 2009)** was applied to study the influence of factors affecting the variation of milk yield and SCS. Two different models were considered. The model equation (1) was used for milk yield:

$$y_{ijklmn} = \mu + Y_i + L_j + M_k + C_l + M_kC_l + u_m + e_{ijklmn} \quad (1)$$

where:

- y_{ijklmn} – individual observations of milk yield
- μ – general mean
- Y_i – fixed factor of year class (2016, 2017, 2018); $\sum_i Y = 0$
- L_j – fixed factor of lactation number (1, 2, 3); $\sum_j L = 0$
- M_k – fixed factor of month in milk (2, 3, 4, 5, 6); $\sum_k M = 0$
- C_l – fixed factor of SCC class (5 levels as mentioned above); $\sum_l C = 0$
- M_kC_l – fixed factor of interaction between month in milk and SCC class; $\sum_{kl} MC = 0$
- u_m – random factor of ewe (1, 2 to 61); $u_m \sim N(0, I\sigma_m^2)$
- e_{ijklmn} – random error; $e_{ijklmn} = N(0, I\sigma_e^2)$

The model equation (2) was used for SCS:

$$y_{ijklmn} = Y_i + L_j + M_k + C_l + M_kC_l + u_m + e_{ijklmn} \quad (2)$$

where:

- y_{ijklmn} – individual SCS
- μ – general mean
- Y_i – fixed factor of year (2016, 2017, 2018); $\sum_i Y = 0$
- L_j – fixed factor of lactation number (1, 2, 3); $\sum_j L = 0$
- M_k – fixed factor of month in milk (2, 3, 4, 5, 6); $\sum_j MIM = 0$
- C_l – fixed factor of milk yield class (5 levels as mentioned above); $\sum_l C = 0$
- M_kC_l – fixed factor of interaction between month in milk number and milk yield class; $\sum_{kl} LMIM = 0$
- u_m – random factor of ewe (1, 2 to 61); $u_m \sim N(0, I\sigma_m^2)$
- e_{ijklmn} – random error; $e_{ijklmn} = N(0, I\sigma_e^2)$

Fixed factors included in the models (1) and (2) were estimated using the Least Squares Means (LSM) method. Statistical significances of fixed factors were tested by Fischer's F-test; statistical significances of individual differences between estimated levels of fixed factors were tested by Scheffe's multiple-range tests. Differences were considered statistically significant when p <0.05 or p <0.01. Ewe and residual error variances were estimated using the Restricted Maximum Likelihood (REML) method. Estimated variances enable to estimate repeatability of MY and SCS and can be interpreted as the proportion of total variance attributable to within-individual variance:

$$r^2 = \frac{\sigma_m^2}{\sigma_m^2 + \sigma_e^2}.$$

RESULTS AND DISCUSSION

Analysis of variance of fixed factors affecting milk yield (MY) and (SCS) of Lacaune (LC) ewes is given in Table 1. The factors of year of measurement (three years included to increase number of observations), lactation number and month in milk (MIM) were significant (p <0.05 or p <0.01). Both, the factor of somatic cell count (SCC) class when MY as dependent variable was analysed and the factor of MY class when SCS as dependent variable was analysed, were significant (p <0.01). The factor of interaction between MIM and SCC class (model 1) was significant (p <0.01). The factor of interaction between MIM and MY class (model 2) was non-significant (p >0.05). Differences in studied traits with respect to individual levels of factors included in models are discussed below.

Table 1 Analyses of variance (statistical significance of Fisher F-test) for milk yield and somatic cell score.

	Traits	
Factor	**MY**	**SCS**
Year	$^{++}$	$^{+}$
Lactation number	$^{+}$	$^{+}$
MIM	$^{++}$	$^{+}$
SCC class	$^{++}$	N.C.
MY class	N.C.	$^{++}$
MIM*SCC class	$^{+}$	N.C.
MIM*MY class	N.C.	$^{-}$

Note: MY – milk yield, SCS – somatic cell score, MIM – month in milk, SCC – somatic cell count, N.C. – not considered, ^{++}p <0.01, ^{+}p <0.05, ^{-}p >0.05.

Table 2 Least squares means and standard errors for milk yield by somatic cell count class and for somatic cell score for somatic cell score by milk yield class.

	SCC class (10^3 cells.ml^{-1})				
	Lowest (1)	**Low (2)**	**Middle (3)**	**High (4)**	**Highest (5)**
	≤200	>200≤400	>400≤600	>600≤1000	>1000
	n=10	n=57	n=138	n=142	n=30
Trait	$\mu \pm s_\mu$	$\mu \pm s_\mu$	$\mu \pm s_\mu$	$\mu \pm s_\mu$	$\mu \pm s_\mu$
MY (ml)	647 ±23	561 ±27	573 ±37	487 ±39	538 ±24
Scheffe's tests			1:2^{+},3^{++},4^{++}		
	MY class (ml)				
	Lowest (1)	**Low (2)**	**Middle (3)**	**High (4)**	**Highest (5)**
	≤200	>200≤400	>400≤600	>600≤1000	>1000
	n=10	n=57	n=138	n=142	n=30
Trait	$\mu \pm s_\mu$	$\mu \pm s_\mu$	$\mu \pm s_\mu$	$\mu \pm s_\mu$	$\mu \pm s_\mu$
SCS	6.69 ±0.83	6.43 ±0.36	5.21 ±0.27	4.95 ±0.27	3.97 ±0.47
Scheffe's tests			2:3^{+},4^{++},5^{++}		

Note: MY – milk yield, SCS – somatic cell score, SCC – somatic cell count, ^{++}p <0.01, ^{+}p <0.05.

Table 3 Least squares means and standard errors for milk yield and somatic cell score by year of measurement, lactation number and month in milk.

		Traits	
Factor		**MY (ml)**	**SCS**
Year	n	$\mu \pm s_\mu$	$\mu \pm s_\mu$
2016 (1)	138	556 ±28	5.62 ±0.31
2017 (2)	138	602 ±24	4.91 ±0.31
2018 (3)	101	526 ±28	5.72 ±0.35
Scheffe's tests		2:3^{++}	2:3^{++}
Lactation number			
First (1)	145	517 ±26	5.90 ±0.33
Second (2)	127	584 ±25	4.80 ±0.30
Third (3)	105	583 ±29	5.64 ±0.35
Scheffe's tests		1:2^{+}	1:2^{+}
Month in milk			
30-60 days (2)	55	733 ±33	5.71 ±0.37
61-90 days (3)	81	667 ±27	6.19 ±0.33
91-120 days (4)	86	565 ±29	5.29 ±0.32
121-150 days (5)	86	454 ±28	4.99 ±0.33
>151 days (6)	69	389 ±29	5.07 ±0.34
Scheffe's tests		2:4^{++},5^{++},6^{++} 3:5^{++},6^{++} 4:5^{+},6^{+}	2:4^{+}

Note: MY – milk yield, SCS – somatic cell score, n – number of observations, ^{++}p <0.01, ^{+}p <0.05.

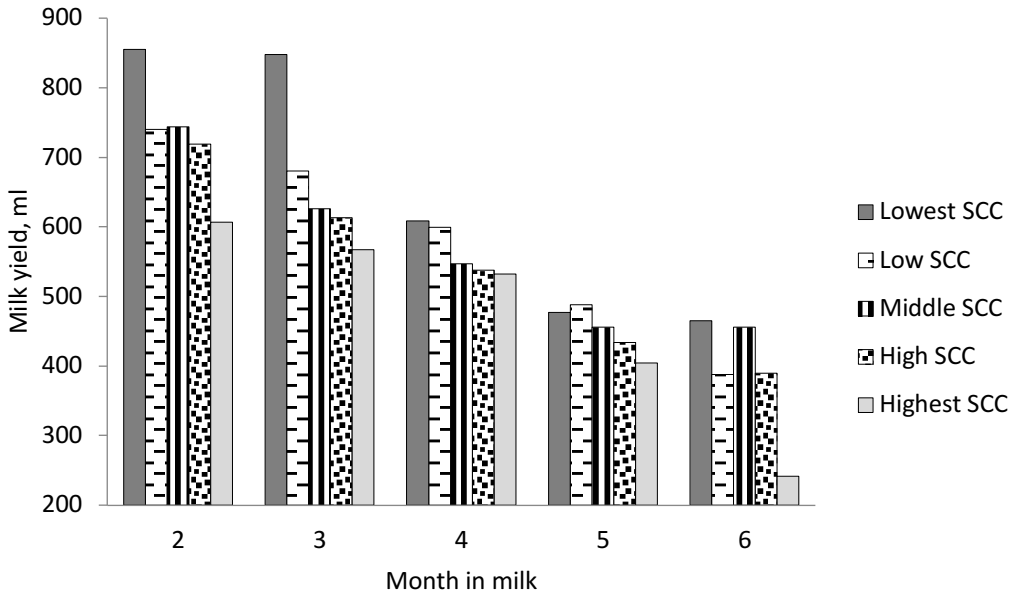

Figure 1 Milk yield in dependence of month in milk and somatic cell count class.

Least squres means (LSM) of MY and SCS confirmed negative relations between these traits (Table 2) i.e. the higher MY, the lower SCS is found. With increasing SCC (model 1), MY deacreased, with exception between classes with SCC>600≤1000 and >1000×10^3 cells.mL^{-1}. The differences between these classes, however, were found non-significant and respective LSM are probably affected by distribution of observations and their lower number (especially in highest SCC class). Accordingly, SCS increased with decreasing MY (model 2). Some differences between individual levels of MY class were also found non-signignicant. The proportion of highest SCC class of i.e. SCC above 1000×10^3 cells.mL^{-1} was 8 % of milk records. The proportion of records with SCC under or equal to 200×10^3 cells.mL^{-1} (lowest class of SCC) was only 3 %. The most of records fell in classes with SCC>400x10^3≤600x10^3 cells.mL^{-1} (middle SCC class) and SCC>600x10^3≤1000x10^3 cells.mL^{-1} (higher SCC class) i.e. 37 % per each. The remaing proportion (15 %) fell in class with SCC>200x10^3≤400x10^3 cells.mL^{-1} (lower SCC class). According to these findings, about 90 % of ewes had healthy udders (or may be of subclinical mastitis udders) as compared with report of **Gonzalo et al. (1994)**, who recommended SCC values ranging from 500×10^3 to 1000×10^3 cells.mL^{-1} as thresholds between healthy and infected udders. When comparing with reports **El-Saied, Carriedo and San Primitivo (1998)**, **Caboni et al. (2017) and Kern et al. (2013)** who recommended SCC values ranging from 250×10^3 to 300×10^3 cells.mL^{-1} as thresholds of healthy udders, the proportion of ewes those could suffer from subclinical mastitis icreased. Regarding distribution of ewes in dependence on SCC class, **Tvarožková et al., (2019)**, who analysed Tsigai, Lacaune and Slovak Dairy breed ewes, reported the following frequencies: about 88 % in lowest class of SCC (under or equal to 200×10^3 cells.mL^{-1}) and about 8 % in highest class of SCC (above 1000×10^3 cells.mL^{-1}) in 2017. Frequencies in 2018 were found to differ: about 21 % and 32 % in lowest and highest class, respectively. High changes between years were probably due to fact that more heterogeneous data were studied (various breeds and various flocks) than data analysed in this study. These also might indicate differences in managment between 2017 and 2018. **Idriss et al. (2015)**, reported highest proportion of ewes in lowest class of SCC and lowest proportion of ewes in highest class of SCC. These proportions slightly differed between breeds (Tsigai, Improved Valachian and Lacaune and their crossbreds, although the same pattern was was found in dependence of breed.

When comparing estimated changes in MY according to SCC class found in this study, these were between 12 and 25 %. **Tančin et al., (2019)**, who also investigated relationships between MY and SCC in Lacaune breed, estimated these changes between 10 to 18 %. When investigated these changes on farm level (five farms), these changes were higher **(Tančin et al., 2017)**. **Sutera et al. (2018)** reported that estimated losses in MY according to SCC level used were about 16% at maximum (in Valle del Belice ewes studied). Although no analyses of microrganisms in udders were done, the negative effect of increased SCC level on milk yield could be supposed when comparing with literature. For example, **Martí De Olives et al. (2013)**, who performed the bacteriological analysis in Manchega ewes, found that milk yield between healthy and infected ewes differred by about 17 % in favour of healthy ewes.

The fluctuation in LSM of MY (also of SCS) in dependence on year of measurement (Table 3) may indicate some problems in management practice of flock, especially when evaluating these traits between 2017 and 2018; probably worse conditions occurred in 2018. A rough increase of MY (and decrease of SCS) were found with increasing lactation number, although some differences were found non-significant (significant difference between first and second lactation was found). The effect of MIM showed significant influence on MY

and SCS although variation of SCS was lower (less significant differences revealed) in comparison to variation of MY (more significant differences revealed). Finding about influence of lactation number partly agreed with previous studies **(Oravcová et al., 2006; Oravcová, Mačuhová and Tančin, 2018)**, who reported no significant differences in MY in dependence on lactation number when LC, Tsigai (TS) and Improved Valachian (IV) ewes and LCxTS and LCxIV crosses were analysed. For log_{10}SCC, the latter authors revealed all differences between invidual lactations to be significant. Detailed comparisons of MY in this study and study of **Oravcová, Mačuhová and Tančin (2018)** those related LC ewes in the same flocks showed worse levels of flock mangement, also in terms of mastitis control might be supposed: in earlier period (2010-2013) higher MY was observed. **El-Saied, Carriedo and San Primitivo (1998)** reported that lactation number and stage of lactation (could be considered as MIM) significantly affected SCS in Churra ewes. In contrast, **Othmane et al. (2002)** found age of ewe (could be considered as lactation number) and stage of lactation to be non-significant when SCC in ewes of the same breed were analysed later. According to the latter authors, no differences were a result of strict mastitis control (teat dip after milking, selective dry therapy and culling of ewes with chronic mastitis) and high levels of husbandry applied in flocks investigated. The lower variation of SCS and higher variation of MY in dependence on stage of lactation was reported for French Lacaune ewes **(Barillet et al., 2001)**.

The influence of interaction between MIM and SCC (Figure 1) was significant when MY (model 1) was analysed. Within individual months, some significant differences between SCC classes were revealed. However, most of differences were found between lower SCC classes (mostly MIM 2 and MIM 3) on the one hand and higher SCC classes (mostly MIM 4, MIM 5 and MIM 6) on the other hand. Comparisons with literature could not be done: to our best knowledge, no study which included interaction between MIM and SCC class in similar way was performed. However, a relationship between lactation stage and comatic cells showed that milk yield seemed to be of the higher influence on SCC at the end of lactation (MIM 6) than at the beginning, which is in accordance with findings of **Arias et al. (2012)**.

Figure 1

When interation between MIM and MY class was considered when SCS (model 2) was analysed, the differences were non-significant, although trends were similar to those found when individual MIM and MY classes were investigated (not shown).

CONCLUSION

The findings of this study confirmed fact that somatic cells were present in ewe milk and may used to indicate udder health and contribue to improve levels of management, in terms of preventing the mastitis to be spread. Because number of somatic cells increases when infectious agents enter the udder, further research aimed at relationships between somatic cells, microorganisms and quality of ewe milk is needed.

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ANTIOXIDANT PROPERTIES OF PROCESSED CHEESE SPREAD AFTER FREEZE-DRIED AND OVEN-DRIED GRAPE SKIN POWDER ADDITION

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ABSTRACT

Processed cheese spread (PCS) is a popular product with high nutritional value and containing protein, fat and minerals. Grape skin is waste from winery processing plants that still has phenolic substances with significant antioxidant activity that could be used for valorisation of processed cheese and increasing the content of nutrients, phenolics and overall antioxidant properties. Both oven-dried (OD) and freeze-dried (FD) grape skin (GS) powder was characterised by the principal ingredients, the content of phenolic compounds and antioxidant capacity. Similarly, the influence of the addition of OD-GS and FD-GS powders on processed cheese spread (PCS) at 1% and 2% (w/w) levels were examined. The OD-GS and FD-GS powders were characterised by protein content, fat content, moisture and dietary fibre, thus showing that drying technique did not affect those parameters. The OD-GS powder exhibited higher content of rutin, (+)-catechin, (-)-epicatechin and total flavonoid content (TFC), while higher total phenolic content (TPC) and ABTS radical cation were observed for freeze-dried GS powder. Fortification of PCS with 1% and 2% (w/w) of GS powder increased protein content. An ANOVA procedure revealed that addition of FD-GS powder to processed cheese spread was superior to TPC values together with rutin, (+)-catechin, and (-)-epicatechin contents. The higher phenolic contents reflected the higher antioxidant capacity of PCS samples fortified with FD-GS powder. Freeze-dried grape skin powder was the better choice for valorisation of processed cheese spread.

Keywords: grape; valorisation; processed cheese; antioxidant; chromatography

INTRODUCTION

Processed cheese spread (PCS) is a multi-component mixture made from water, cheese, fat, and emulsifying salts (phosphates or citrates). This mix is processed by stirring and melting in temperatures ranging from 85 to 110 °C for up to 20 min **(Černíková, et al., 2018)**. The obtained hot mixture is poured into cups, cooled down and stored at refrigeration temperature. Processed cheeses are products with extended shelf life that deliver bioactive proteins, fats, minerals and vitamins to consumers **(Henning, et al., 2006)**. Despite the high nutritional value, various types of cheese have been enriched by addition of herb or medicinal plant extracts during their preparation, for instance, the addition of rosemary leaves to ripened semi-hard cheese **(Marinho, et al., 2015)**. The fortification of frequently used food products may enhance the consumption of various health-promoting substances and might be helpful for human health **(Rashidinejad, et al., 2015)**. The authors found that hard low-fat cheese fortified by catechin maintained its antioxidant activity after *in vitro* digestion experiment.

Grape berries (*Vitis vinifera L.*) are used in the winemaking industry to produce alcoholic beverages by pressing berries and subsequent fermentation of liquid. The press residues constitute 20% (w/w) of the total grapes used for wine production **(Teixeira, et al., 2014)**. Grape pomace from white grape varieties is an excellent source of phenolic compounds (for example gallic acid, catechin, epicatechin and procyanidins) **(Genova, Tosetti and Tonutti, 2016)**; therefore, it can be used for the valorisation of various food products. Grape skin powder or grape flour has been successfully incorporated into bread **(Šporin, et al., 2018)** or yoghurts **(Karnopp, et al., 2017)**. Only a limited number of studies regarding the enrichment of processed cheese, probably due to the higher processing temperature and high-fat content, exist. In our recent studies, we described the effect of processing parameters on the antioxidant properties of processed cheeses fortified by quercetin or/and rutin **(Přikryl, et al., 2018)**. Functional processed cheese spreads have been prepared with the addition of tomato paste **(Mehanna, et al., 2017)**, carrot paste **(Mohamed, Shalaby and Gafour,**

2016) and pulp from apricots **(Mohamed and Shalaby, 2016)**. In a study of **Torri et al. (2016)**, grape skin powder was added to cow's milk curd before ripening process to produce Robiola (soft-ripened cheese), focusing on optimisation of enrichment using sensory evaluation. The authors found that the amount of powders exceeding 0.8% and 1.6% (w/w) for Barbera and Chardonnay powder, respectively, negatively affected the acceptability of cheeses. In a recent study, various fruit and vegetable by-products were added into the curd to produce Primosale cheese **(Costa, et al., 2018)**. They found wine pomace to be an excellent source of polyphenolic compounds with antioxidant activity. The main aim of this study is to determine the effect of the addition of grape skin powder on principal ingredients and antioxidant properties of processed cheese. We assumed that the addition of freeze-dried grape skin powder would enhance the functional properties of enriched processed cheese spread.

Scientific hypothesis

Freeze-dried grape skin powder enhances the antioxidant status of fortified processed cheese spread more than the oven-dried grape skin powder does.

MATERIAL AND METHODOLOGY

All solvents for extraction, chromatographic analysis and chemicals used for determination of antioxidant activity were purchased from Sigma-Aldrich (Prague, Czech Republic).

Preparation of grape skin powder

Grapes of the white variety 'Müller Thurgau' were harvested from the Prostřední Hory (Bzenec, Czech Republic) vineyard track during September 2017. After pressing the grape berries to obtain a liquid for wine manufacturing, a portion of grape pomace was immediately stored at -20 °C in an evacuated plastic package. Before processing, grape pomace was thawed and the needles and seeds were removed using an analytical sieve (mesh size 0.5 × 0.5 cm). Grape skins (GS) were dried at the following conditions: oven-drying (OD) was performed in laboratory air-forced oven (HS62A, Chirana, Brno, Czech Republic) at 46 °C for 24 h. Freeze-dried (FD) samples were prepared at -40 °C (12 Pa) for 48 h (CoolSafe 100-4, Trigon Plus, Čestlice, Czech Republic). Dried grape skins were milled at 5000 rpm for 10 s with a Grindomix GM 200 (Retsch GmbH, Haan, Germany) and sieved to obtain particles <800 μm. Both GS-OD and GS-FD powders were stored in a tightly sealed plastic pack at -20 °C until use. The contents of crude protein (Method 960.52), fat (Method 920.39), moisture (Method 934.01), ash (Method 930.05) and total dietary fibre (Method 985.29) were determined according to AOAC **(Horwitz, 2000)** procedure in duplicate. The total content of saccharides was calculated from differences.

Processed cheese manufacturing

The composition of raw materials, including Eidam cheese (dry matter ≈50 g.100 g^{-1} and fat in dry matter ≈30 g.100 g^{-1}, 8-week maturity), butter (dry matter ≈84 g.100 g^{-1} and fat in dry matter ≈98 g.100 g^{-1}) and water, was adjusted to obtain processed cheese with dry matter ≈37 g.100 g^{-1} and fat in dry matter ≈50 g.100 g^{-1}. A ternary mixture of monosodium dihydrogenphosphate (19%), disodium hydrogen phosphate (37%), tetrasodium diphosphate (22%) and the sodium salt of polyphosphate (22%) was used in a total concentration of 2.8 g.100 g^{-1}. For the preparation of functional processed cheese spread (PCS), OD-GS and FD-GS powders were added to produce PCSOD-GS and PCSFD-GS samples. Both grape skin powders were added at 1.0 and 2.0% (w/w) levels. Processed cheese without grape skin powder served as a control. Model processed cheese was manufactured in Stephan UMC-5 (Stephan Machinery GmbH, Halmen, Germany) equipment with indirect heating as is described in the flow chart below (Figure 1). Eidam block cheese and butter were cut into small pieces, put into the kettle and minced for 30 s. Then water, emulsifying salts and GS powder were added into the blend. The mixture was heated at 90 °C for 13 min at a constant agitation of 1500 rpm. Samples were poured into 80 g polystyrene doses with caps. The packages were cooled down to 6 °C and stored at -20 °C to avoid deterioration. Dry matter (DM), fat and protein contents were evaluated according to Method 969.19, Method 2001.14 and Method 960.52 as described in **Horwitz (2000)**, respectively, in three repetitions.

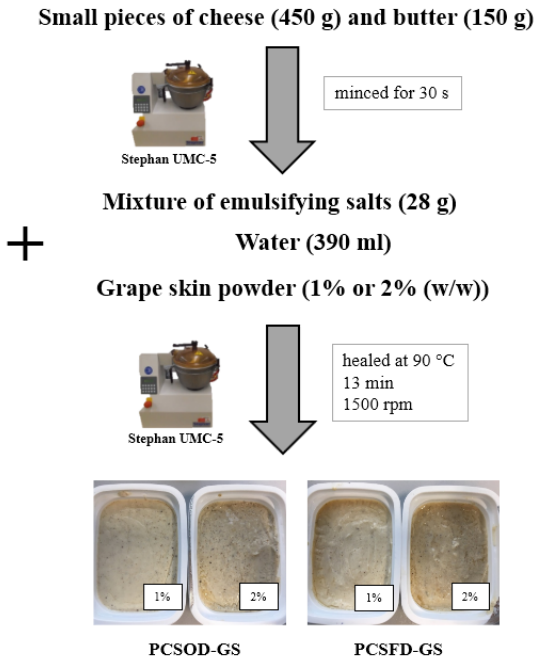

Figure 1 Flow chart of the cheese-making process. PCSOD-GS = processed cheese spread with oven-dried grape skin powder, PCSFD-GS = processed cheese spread with freeze-fried grape skin powder.

Extracts preparation

A glass test tube with 1.0 g of dried GS sample and 10.0 mL of 50% methanol solution was put in ultrasonic bath Sonorex TK52 (Bandelin Electronic, Berlin, Germany) for 30 min. A clear supernatant after centrifugation at 4100 rpm for 5 min (Universal 320, Hettich, Tuttlingen, Germany) was obtained.

Extract of PCS samples was obtained according to the procedure described in **Přikryl et al. (2018)**, i.e. 1.0 g of frozen PC sample was extracted into 10.0 mL of 50% methanol solution in an ultrasonic bath for 30 min. Subsequently, elimination of proteins and salts was performed using the procedure of **Khalifa, Omar and Mohamed (2017)** with a slight modification; the pH of extract was adjusted to 4.0 using HCl (2 M) and precipitated proteins were removed by centrifugation at 6000 rpm for 10 min. Then, the pH of clear supernatant was adjusted to 7.0 using NaOH (1 M) followed by centrifugation at 6000 rpm for 10 min to remove remaining proteins and salts. Supernatants were kept refrigerated and used for antioxidant assays and HPLC analysis. Two extracts per sample were prepared, and each extract was measured in duplicate, resulting in a sample size N = 4.

Chromatographic analysis of the extracts (HPLC analysis)

First, GS were screened for the presence of the following phenolic compounds: quercetin, rutin, (+)-catechin, (-)-epicatechin, resveratrol, caffeic acid, p-cumaric acid and ellagic acid. Secondly, phenolic substances identified in GS extracts were determined in PCSOD-GS and PCSFD-GS extracts. Before injection, each extract was filtered through a syringe filter (0.45 μm, Labicom, Olomouc, Czech Republic). Samples were injected into an Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped with a degasser, an autosampler, a binary pump, a thermostated column compartment and DAD detector. A Zorbax Elipse Plus 1.8 μm C18 (50 × 2.1 mm; Agilent Technologies) column thermostated at 40 °C was used. For the analysis, 2 μL of the sample were injected. A mixture of 0.01 M ammonium acetate adjusted to pH 3.1 using formic acid (solution A) and acetonitrile (solution B) was used as mobile phase with a flow rate 0.6 mL.min^{-1}. The gradient for solution B was 0-3 min at 3%; 10 min at 20% and 20 min at 80%. The signal was detected at 280 nm. The identification of each peak in chromatograms of the extracts was carried out by comparing retention time and absorption spectrum against a pure standard. Quantitative determinations were done using calibration plots of a selected external standard.

Determination of antioxidant properties

Total phenolic content (TPC) was determined by measuring the complex of antioxidants with Folin-Ciocalteau reagent at 765 nm (DU 530, Beckman Coulter Inc., Brea, USA) using the procedure described in **Přikryl et al. (2018)**. The results were expressed as gallic acid (GAE) equivalents (mg.g^{-1} of dry matter (DM)). Total flavonoid content (TFC) was determined using aluminium chloride assay **(Denni and Mammen, 2012)**. The increase of absorbance at 415 nm was proportional to the increase in the content of flavonoids. Results were reported as quercetin (QUE) equivalents (mg.g^{-1} DM).

ABTS (2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging activity assay was adopted from our previous study **(Červenka, et al., 2018)**. The reaction between ABTS$^{\bullet+}$ and antioxidants was monitored at 734 nm, and the results were reported as Trolox equivalents antioxidant capacity (TEAC) in mg.g^{-1} DM.

Reducing power (RP) of extracts was determined via the formation of Prussian blue at 700 nm (ferric-ferrous complex) according to the procedure of **Pavithra and Vadivukkarasi (2015)**.

Statistical analysis

Order statistic methods for small sample size were used throughout this study. The mean and its deviation were calculated according to Horn's procedure **(Horn, 1983)** and were expressed as pivot half sum (P_L) and pivot range (R), respectively. Nonparametric statistical methods were used in this study. The sign test was applied for pair-wise comparisons between means for grape powder samples. As far as processed cheese is concerned, the pair-wise comparison procedure was performed using Tukey's method. A two-factor Kruskal-Wallis analysis of variance (ANOVA) was applied to determine whether the drying method of GS powder (factor A) and the amount added to PCS sample (factor B) influenced the antioxidant properties in fortified PCS samples. To determine associations among variables, Spearman rank-order correlation coefficients (r) were assessed. All the statistical treatments were done at the probability P = 95%) (Statistica CZ, 12.0, StatSoft CR s.r.o., Prague).

RESULTS AND DISCUSSION

Composition of grape skin powder

As can be seen from Table 1, there were no statistical differences between the contents of crude protein, fat, total dietary fibre and ash for oven-dried and freeze-dried GS powders. On the other hand, the type of drying process affected the TPC and antioxidant properties of samples. Freeze-dried GS powder has a higher level of TPC (19.97 ±1.60 mg GAE.g^{-1} DM) and more than twice as high $TEAC_{ABTS}$ value (127.10 ±27.28 mg Trolox.g^{-1} DM) in comparison with the oven-dried GS samples. It has been previously concluded that the freeze-drying process is superior for the preservation of antioxidant compounds, and probably higher efficiency of extraction due to pronounced disruption of plant cells via the formation of ice crystals **(Kamiloglu, et al., 2016, Kamiloglu and Capanolgu, 2014)**. However, such a release of flavonoid compounds from plant cells may cause their pronounced exposure to oxygen. As was described in a study of **Nunes et al. (2016)**, oven drying of guava powder released more soluble flavonoids than the freeze-drying technique. In the chromatogram (Figure 2) of dried grape skin powder extract, fourteen well-resolved peaks were observed. However, from eight selected polyphenolic compounds, only the presences of (+)-catechin, (-)-epicatechin and rutin were confirmed in grape skin powder samples using retention time and absorption spectra in the current study. There was no difference in the number of peaks/compounds in oven-dried and freeze-dried GS powder extracts. The chromatograms differed in the heights of the peak showing that oven-dried GS powder extracts contained more polyphenolic substances.

Table 1 The main composition and antioxidant properties of grape skin powders (pivot half sum (P_L) ±pivot range (R), N = 2).

	Oven-dried	Freeze-dried
Main composition (g.kg^{-1})		
Protein	157.5 ±7.0^{A}	150.5 ±4.2^{A}
Fat	74.2 ±5.1^{A}	72.3 ±4.5^{A}
Moisture	55.0 ±4.3^{A}	59.5 ±6.4^{A}
Ash	13.2 ±2.0^{A}	14.6 ±3.1^{A}
Total saccharides	701.2	703.5
Total dietary fibre	228.8 ±8.5^{A}	231.7 ±9.1^{A}
Phenolic content (mg.g^{-1} DM)		
(+)-catechin	1712.5 ±1.2^{B}	1450.0 ±1.5^{A}
(-)-epicatechin	1383.3 ±3.0^{B}	1023.4 ±2.2^{A}
Rutin	221.8 ±1.0^{B}	112.0 ±1.2^{A}
Total phenolics (mg GAE.g^{-1} DM)	10.1 ±0.93^{A}	19.97 ±1.60^{B}
Total flavonoids (mg QUE.g^{-1} DM)	0.73 ±0.01^{B}	0.52 ±0.02^{A}
Antioxidant activity		
$TEAC_{ABTS}$ (mg Trolox.g^{-1} DM)	54.44 ±2.44^{A}	127.10 ±27.28^{B}
Reducing power (absorbance unit)	1.140 ±0.010^{A}	1.136 ±0.012^{A}

Note: DM, dry matter; GAE, gallic acid; QUE, quercetin; $TEAC_{ABTS}$, Trolox equivalent antioxidant capacity using 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid. Values sharing the same superscript letters in row $^{(A-B)}$ are not statistically significant different from each other (the sign test, p <0.05).

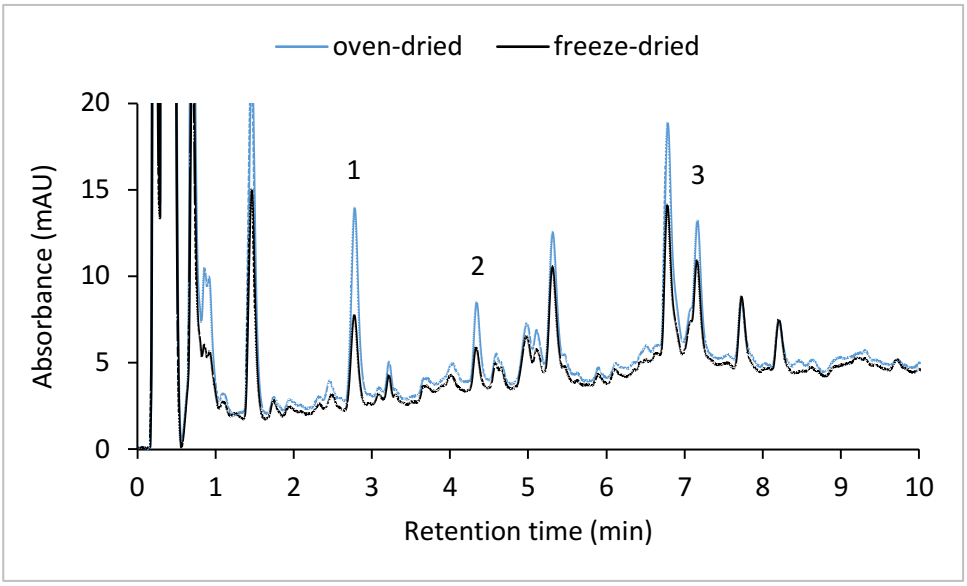

Figure 2 Separation of phenolic compounds in oven-dried and freeze-dried grape skin powder extracts using HPLC/DAD, detection at 280 nm; (+)-catechin (**1**), (-)-epicatechin (**2**) and rutin (**3**).

As shown in Table 1, significantly higher contents of (+)-catechin, (-)-epicatechin and rutin were observed in oven-dried GS powders, which corresponded to TFC values.

Properties of processed cheese spread containing grape skin powder

Table 2 shows the differences in the main composition of fortified processed cheese. The addition of grape skin powders significantly increased the protein content in processed cheese samples when added in 2% (w/w) levels, i.e. from 112.5 ±5.0 g.kg^{-1} (in control) to 128.4 ±5.7 and 129.7 ±2.3 g.kg^{-1} for processed cheese enriched with freeze-dried and oven-dried GS powders, respectively (p <0.05). Since we used the same amount of ingredients (cheese, butter, polyphosphate salts) for the preparation of all of the processed cheese samples, the variation in protein content was due to the addition of grape skin powder. Although the values of dry matter content increased linearly with the level of addition of grape skin powder, the effect of fortification was recognized as insignificant (p >0.05). On the contrary, **Khan et al. (2018)** found that fortification of Gouda cheese with the mango kernel did not significantly change the protein content.

Pair-wise comparison tests revealed that only processed cheese with 2% (w/w) of oven-dried GS powder had significantly higher dry matter content than that found in

Table 2 The main composition of processed cheese spread (PCS) fortified with oven-dried (OD) and freeze-dried (FD) grape skin powder (GS). Pivot half sum (P_L) ±pivot range (R) (N = 3).

Ingredient (g.kg^{-1})	PCS control	PCS fortified with OD-GS powder (%, w/w)		PCS fortified with FD-GS powder (%, w/w)	
		1.0	2.0	1.0	2.0
Protein	112.5 ±5.0^{A}	120.3 ±4.3^{A}	129.7 ±2.3^{B}	118.0 ±3.8^{A}	128.4 ±5.7^{B}
Fat	191.0 ±3.1^{A}	188.5 ±2.7^{A}	187.4 ±3.0^{A}	189.3 ±4.2^{A}	186.9 ±1.8^{A}
Dry matter	368.2 ±14.0^{A}	394.6 ±6.2^{AB}	419.0 ±18.0^{B}	387.3 ±7.0^{AB}	395.9 ±4.8^{AB}
Ash content	39.5 ±1.8^{A}	40.8 ±2.2^{A}	42.1 ±1.4^{A}	40.9 ±1.1^{A}	42.4 ±2.0^{A}

Note: Values sharing the same superscript letters in row $^{(A-B)}$ are not statistically significant different from each other (Tukey's pair-wise comparison test, p <0.05).

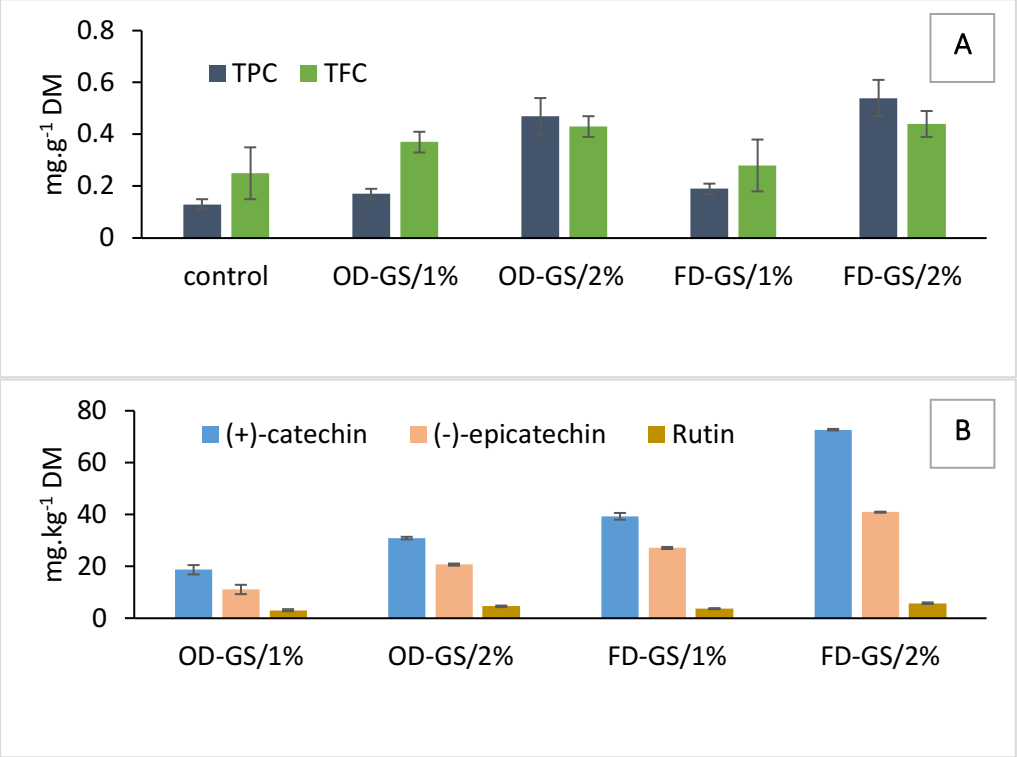

Figure 3 The content of **A**) total flavonoid (TFC), total phenolic contents (TPC), and **B**) phenolic individuals in processed cheese spread supplemented with oven-dried (OD) and freeze-dried (FD) grape skin (GS) powder at 1% and 2% (w/w) levels. Results are expressed as gallic acid and quercetin equivalents for TPC and TFC, respectively.

control (p <0.01). Fat and ash contents remained similar for all the processed cheese samples (p >0.05).

Antioxidant properties of processed cheese spread containing grape skin powder

The content of phenolic individuals is depicted in Figure 3B and show their higher content in processed cheese fortified with freeze-dried grape skin powder at both levels. Total phenolic content of processed cheese samples was determined using Folin-Ciocalteau's assay (Figure 3A). Significant increase of TPC values was obtained for processed cheese supplemented with GS powder at 2% (w/w) level from 0.13 ±0.02 mg GAE.g^{-1} DM (control sample) to 0.47 ±0.07 and 0.54 ±0.07 mg GAE.g^{-1} DM for oved-dried and freeze-dried GS powder, respectively. The addition of OD-GS powder to processed cheese spread resulted in the increase in TFC values from 0.25 ±0.10 mg QET.g^{-1} DM (control sample) to 0.37 – 0.43 mg QET.g^{-1} DM without respect to GS level. Freeze-dried GS powder enhanced processed cheese samples with flavonoids at a higher level (2%, w/w). It is interesting to note that even the control sample exhibited TFC value that then slightly increased with the addition GS powder. It has been reviewed that antioxidant properties of milk and milk products are due to the presence of sulphur-containing amino acids, vitamins, enzymes, peptides and oligosaccharides **(Khan, et al., 2019; Usta and Yilmaz-Ersan, 2013; Atmaca, 2004; Egger and Ménard, 2017; Alenisan, et al., 2017)**. Although oven-dried GS powder exhibited higher levels of (+)-catechin, (-)-epicatechin and rutin, their content in PCS samples with OD-GS powder decreased in comparison with PCS fortified with FD-GC powder. TPC values showed very high correlation with the content of all phenolic constituents (0.746 < r < 0.826, p <0.01) while low correlation coefficients have been

observed for TFC values showed values where (r = 0.610, p <0.05).

Antioxidant capacity of processed cheese samples in terms of $TEAC_{ABTS}$ significantly increased with the increase of GS level (p <0.05). Surprisingly, $TEAC_{ABTS}$ values were approximately tenfold higher (0.31 – 0.72 mg Trolox.g^{-1} DM) for processed cheese with 1% (w/w) of GS in comparison with that of the control, followed by an additional tenfold increase (5.22 – 6.69 mg Trolox.g^{-1} DM) when 2% (w/w) of GS powders were added (Figure 4). Such an increase may be attributed to the higher temperature used for melting the processed cheese in this study (90 °C for 13 min). It was previously published that pasteurization enhanced antioxidant properties of grape juice by releasing some polyphenolic compounds that were previously bound to other molecules **(Genova, Tosetti and Tonutti, 2016; Fuleki and Ricardo-da-Silva, 2003)**. For instant, general increase in ABTS scavenging was detected in fresh and technologically harvested grape juices followed by pasteriuzation at 78 °C for 30 min **(Genova, Tosetti and Tonutti, 2016)**.

In our previous work, the antioxidant properties of processed cheese fortified by rutin or quercetin increased with the increase of melting temperature as measured by ABTS•$^{+}$ assay **(Přikryl, et al., 2018)**. In addition, polyphenolics contributed differently to antioxidant capacity measured by ABTS•$^{+}$, as was observed in a study of **Lingua et al. (2016)**. They found that (-)-epicatechin, peonidin-3-glucoside and peonidin-3-acetylglucoside positively correlated with the assay, while pigment A and siringetin-3-glucoside had a negative effect. Similarly, strong positive correlation was observed for $TEAC_{ABTS}$ values and the content of (+)-catechin, (-)-epicactechin and rutin (r = 0.955, p <0.001; r = 0.739, p <0.01 and r = 0.951, p <0.001, respectively). Considering that high correlation coefficient reflects the strong association between variables, we may conclude that $TEAC_{ABTS}$ was mainly influenced by (+)-catechin and rutin contents followed by TPC values (r = 0.877, p <0.01).

The addition of GS powder had influence on reducing the power of processed cheese samples (Figure 4), where an increase was observed with the increase of GS powder level. Even though oven-dried and freeze-dried GS powders had similar RP values (see Table 1), processed cheese samples enriched with freeze-dried GS powder showed significantly higher RP values than in the case of incorporation of oven-dried GS powder, particularly at the 2% level (w/w) (p <0.05). Those discrepancies can be explained by the formation of new compounds, which may have enhanced the antioxidant capacity of the samples. Although the masking of antioxidant properties of various plant-based extracts by the addition of milk or whey proteins is common in literature, an interaction of protein and polyphenolic compounds may also increase antioxidant capacity. For instance, the mixing of α-casein or β-casein with epigallocatechin gallate led to the increase of inhibition of ABTS•$^{+}$ during storage **(Almajano, Delgado and Gordon, 2007)**. In a study by **Sęczyk, Świeca and Gawlik-Dziki (2017)**, the addition of green coffee extract into soymilk significantly elevated ABTS radical scavenging ability (3.5-fold) and reducing power (13.8-fold). The study of interactions between polyphenolic compounds and β-conglycinin revealed that antioxidant capacity increased after the formation of the protein-phenolic complex **(Zhao, et al., 2018; Murray, 2002)**. The authors also demonstrated the increase of antioxidant capacity of the protein-phenolic mixture after heating at 90 °C for 30 min. Thus, we suppose a formation of new products during the preparation of enriched process cheese with enhanced antioxidant activity towards ABTS•$^{+}$. Both TPC and TFC values had the greatest influence on reducing power. Strong positive associations have been found for RP vs. TPC (r = 0.97, p <0.001) and RP vs. TFC content (r = 0.944, p <0.001).

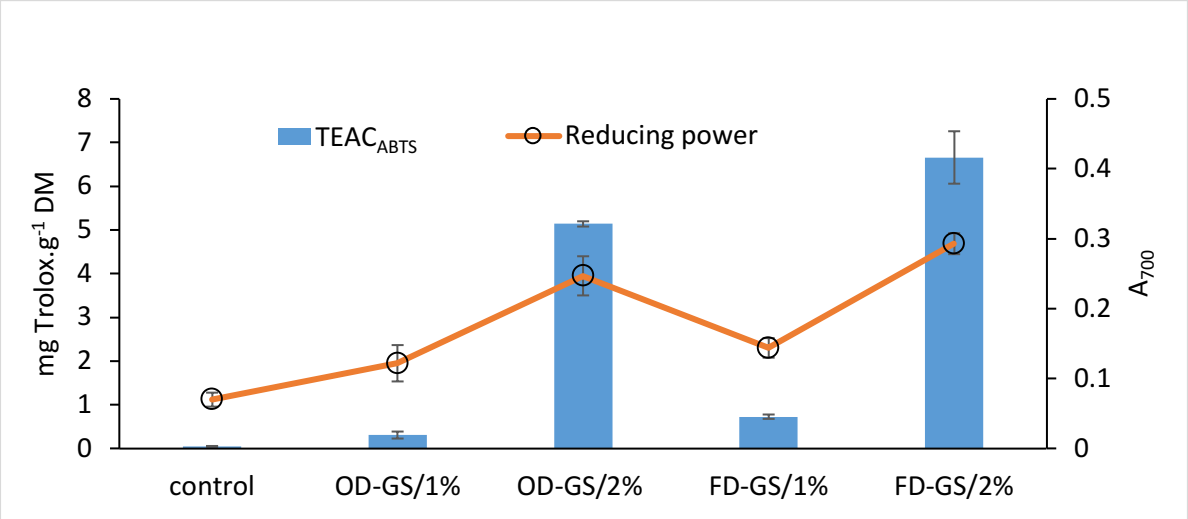

Figure 4 Trolox equivalent antioxidant capacity using ABTS ($TEAC_{ABTS}$, left y-axis) and reducing power (right y-axis) of processed cheese spread supplemented with oven-dried (OD) and freeze-dried (FD) grape skin (GS) powder at 1% and 2% (w/w) levels.

Rutin, (+)-catechin and (–)-epicatechin also showed strong positive correlations with reducing powder (p <0.001) of processed cheese enriched with GS powder, i.e. r = 0.859; 0.842 and 0.917, respectively.

Two-factor Kruskall-Wallis ANOVA was applied to study the effect of grape skin powder levels and type of drying used for their preparation (oven-dried vs. freeze-dried). As can be seen from Table 3, the drying technique has a significant effect on the TPC values, all the phenolic individuals, and reducing power (p <0.01) in processed cheese spreads where the addition of freeze-dried GS powder assured their higher values. Total flavonoids and $TEAC_{ABTS}$ values were not influenced by the drying technique. The effect of GS powder level was found to be significant for all parameters except for (-)-epicatechin content. As expected, the addition of GS powder at a higher level was reflected in higher values of TPC and TFC values, rutin and (+)-catechin contents, and antioxidant capacities in terms of $TEAC_{ABTS}$ and reducing power.

Table 3 The effect of drying technique (Factor A) for preparation of grape skin powder and its amount (Factor B) added to precessed cheese spread using two-factor Kruskall-Wallis ANOVA procedure.

	Factor A	Factor B
Total phenolics	p <0.05	p <0.001
Total flavonoids	p <0.05	p <0.01
(+)-catechin	p <0.01	p <0.001
(-)-epicatechin	p <0.01	p <0.05
Rutin	p <0.001	p <0.01
$TEAC_{ABTS}$	p <0.05	p <0.001
Reducing power	p <0.01	p <0.001

Note: $TEAC_{ABTS}$, Trolox equivalent antioxidant capacity using 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid.

CONCLUSION

Grape skin powder, a waste product from wine production, can be used as an ingredient for the production of processed cheese with enhanced properties. Grape skin powder samples have a high content of protein (150.5 – 157.5 $g.kg^{-1}$) and total dietary fibre (228.8 – 231.7 $g.kg^{-1}$). Freeze-dried grape skin powder possessed higher total phenolic content and the ability to scavenge $ABTS^{\bullet+}$, but lower total flavonoid content as well as the levels of rutin, (+)-catechin, and (-)-epicatechin. The incorporation of grape skin powder at 2 % (w/w) levels into the processed cheese significantly increased the protein content to 128.4 and 129.7 $g.kg^{-1}$ for for freeze-dried and oven-dried grape skin powders, respectively. Addition of freeze-dried grape skin powder into processed cheese was beneficial to antioxidant capacity in terms of reducing power. Higher contents of rutin, (+)-catechin and (-)-epicatechin, as well as total phenolic content, were achieved through the incorporation of freeze-dried GS powder, as was determined using two-factor ANOVA procedure. Based on the chemical analysis, we may conclude that using freeze-dried grape skin powder for the valorisation of processed cheese spread is the better choice in comparison with oven-dried grape skin powder. The hypothesis of this research was confirmed.

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MODELLING OF THE PROCESS OF VYBROMECHANICAL ACTIVATION OF PLANT RAW MATERIAL HYDROLYSIS FOR PECTIN EXTRACTION

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ABSTRACT
Centrifugal and vibrational technological effects are among the main approaches to intensify the process of plant raw materials hydrolysis for pectin extraction. With the impulse intensification of such a process, it is possible not only to increase its efficiency, but also to achieve the compactness of the equipment, reduce the cost of electricity and improve the quality of the product of hydrolysis. The hypothesis is confirmed, according to which the vibro-centrifugal intensification of hydrolysis increases the driving force of the process by not only activating the material flows of raw materials and reagents, but also by reducing the resistance in the technological environment.
Graphical and analytical dependencies of the power and energy parameters of the oscillatory system were obtained, which proved the overcoming of the flow resistance of the liquid medium in the entire speed range of the drive shaft with the potential to intensify the process at a power consumption of 2.0 – 3.0 kW and or by the force of 2.3 – 2.5 kN using the Lagrange and Cauchy methods for composing and solving the equations of motion of the moving components of the tested hydrolyser with vibrating activators, and the methods of mathematical analysis and their processing in the MathCAD. The analysis of the presented parameters of the studied process of mixing the pectin-containing mass in the hydrolyser allowed us to determine the rational mode parameters of processing, which correspond to the angular velocity of the drive shaft $\omega = 150 - 150$ rad/s at the power consumption of 500 – 600 watts.

Keywords: plant raw material; hydrolysis; pectin; vibration motor; vibrator

INTRODUCTION
The process of plant raw material hydrolysis is one of the most common mass exchange processes of food technologies, in particular, the pectin extraction technologies **(Krasnikov et al., 1985)**.

There are different methods of solving the problem of removing solids outside the zone of intense mass exchange in the industry, for example, the use of ultrasound, firehoses for breaking foam, the addition of various surfactants that reduce the surface tension of the liquid. Any defoamers introduced into the solution impair the quality of the solute **(Kolyanovska et al., 2019)**. The intensification of this mass transfer process is implemented through the mixing of raw materials, which requires the study of the motion of the solid phase in the hydrolysate medium, allowing to form the necessary basis for the further theoretical study of the flow patterns and modes of hydrodynamic suspensions motion **(Bubelová et al., 2017; Zverev and Sesikashvili, 2018)**.

Mixing of raw materials in the process of hydrolysis of finely chopped fruits and vegetables for pectin extraction is implemented in the machine with mechanical or pneumatic stirring, with a fluidized bed of technological environment, with the help of the fluid or air jet, in screw and other machines with a moving and stationary solid layer. The mixing units with compressed gas and physical-mechanical volumetric activation methods based on cavitation and vibration effects are becoming widespread. The use of mechanical methods of intensifying the stirring of reagents in hydrolysers is somewhat limited due to the possibility of corrosion damage to metal surfaces and the need to use sufficiently expensive protective coatings or special methods of forming the surface layer of mixers. The erosion and corrosion products of the mixing devices further contaminate pectin, the target hydrolysis product, which is unacceptable **(Krasnikov et al., 1985; Kolyanovska et al., 2019; Czako et al., 2018)**.

Among the various forms of mechanical action on dispersion in technological processes, vibration action is one of the most effective methods for creating and correcting the required dynamic state. The adding of a vibration field on the technological environment significantly activates and intensifies the processes of heat and mass transfer, improves the quality of mixing of materials with different physical and mechanical properties, and helps to reduce the duration of technological operations and energy costs **(Palamarchuk**

et al., 2015; Sukhenko et al., 2017). The vibrating mixing effects allow for a uniform and intense mass exchange between the solid and the liquid phase and have a great potential for energy-saving technologies. Vibrating mixers, compared to conventional ones, have a higher specific performance (5-6 times higher), provide a reduction of mixing time by 2 to 3 times, metal usage by 17 %, power consumption by 30%, capital costs for manufacturing by 18 % and drive power by 30 – 35 %, which results in the decrease of the total energy consumption by 3 – 4 times **(Palamarchuk et al., 2019 a)**.

The process of hydrolysis with such stirring of a suspension of ground plant pectin-containing raw materials in hydrolysers allows increasing the propelling power of the process and energy dissipation in the technological environment. Therefore, the justification of the effective conditions of hydromechanics of the plant raw materials hydrolysis process formation based on the laws of motion of the hydrolyser working parts in the conditions of vibration centrifugal mixing of the hydrolysis suspension study, the saturation of the technological environment with energy that is necessary for the conversion of protopectin into pectin, are relevant problems that are solved in the current scientific research **(Krasnikov et al., 1985; Kolyanovska et al., 2019; Sukhenko et al., 2020)**.

Scientific hypothesis

The research is based on the scientific hypothesis, the main provisions of which are: increasing the driving force of the dissolution process, reducing the cohesive forces of the interaction of the dispersed particles with the blade and increasing the number of equilibrium conditions of the dispersed particles in a liquid dispersive medium, provided that to reduce the resistance in the liquid process mass during mixing, to increase the speed of the blade shaft and to reduce the energy consumption during the operation of the mixer; improve the quality of mixing; to improve the conditions of the bottom of the working area from sediment and to self-clean the working bodies of the sediment.

MATERIAL AND METHODOLOGY

The Lagrange equations of the second kind were used for the theoretical analysis and justification of the power and energy characteristics of the developed machine for hydrolysis of pectin-containing raw materials with vibration centrifugal excitation of the technological environment. The Cauchy method was used for solving these equations. Methods of mathematical analysis and processing in the MathCAD were also applied to obtain the necessary graphical and analytical dependencies of the basic operating parameters of the vibrating system **(MathCAD 12).**

Statistic analysis

Analysis of variance methods was used for the mathematical processing of the results of the experimental studies by means of Microsoft Excel 2013 and the statistical analysis software for crop production "AGROS".

RESULTS AND DISCUSSION

Enabling the oscillatory motion for the moving parts of the tested hydrolyser allows to reduce the resistance of the liquid technological environment during mixing, which, accordingly, reduces the energy consumption during the operation of the stirrer; increases the speed of the blade shaft; increases the degree of mixing; improves the conditions for cleaning of the bottom of the hydrolysers from the sediment; allows to carry out self-purification of the working parts from the sediment by reducing the adhesive forces of the interaction of the dispersed particles with the blade of the agitator and the cohesive forces between the particles of the raw material **(Rachmat et al., 2010; Zheplinska et al., 2019; Palamarchuk et al., 2019 b; Barbaro et al., 2009)**.

Hydrodynamic and mechanical methods for creating a non-equilibrium oscillatory system can be implemented for the equipment under study.

The hydrodynamic method involves the use of a liquid medium as an elastic component of the process, and the periodic change of pressure as a force, which greatly simplifies the regulation of the driving force, reduces the number of actuator elements and, accordingly, increases the reliability of the mechanism. At the same time, the design of the working capacity is complicated because of the need to install guides to ensure periodic braking of the flow and there is an unpredictability of its interaction with the moving fluid from the mixing of the technological environment. It is also necessary to provide an additional hydrodynamic circuit with the pump to ensure the periodic change in fluid pressure complicates the design of the hydrolysers **(Palamarchuk et al., 2013; Ha and Liu, 2009; Burger et al., 2007; Degond and Motsch, 2008.)**.

The mechanical method requires additional introduction into the system of the spring element 2 and the unbalanced unstable elements 3, the specific arrangement of which allows for linear oscillatory motion of the blade shaft 4 and, accordingly, the working blades of the machine in Figure 1.

The main disadvantages of such a model are the increase in dynamic loads on the bearing units 5 and the need to disassemble the mechanism for performing tuning operations, which are not significant enough compared with the disadvantages of the hydrodynamic system **(Nowak and Lewicki, 2004; Palamarchuk, Turcan and Palamarchuk, 2015; Palamarchuk Bandura and Palamarchuk, 2013; Ballerini et al., 2008)**.

To ensure linear oscillatory motion of the blades of the mixer, the model with a combined imbalance of the working elements under the action of circular forcing torque $M_к$ and circular forcing force F_k was used, which causes an increase in dynamic loads on the support units and, at the same time, allows to implement more intensive oscillatory motion **(Palamarchuk Bandura and Palamarchuk, 2013)**.

The presented compelling factors of mechanical unbalance can be determined by the value of the centrifugal force *F* and the moment from the action of two forces in Figure 2:

$$F = m_d \cdot L_d \cdot \omega^2 \quad (1)$$

where: m_d – unbalanced mass; L_d – the distance from the centre of mass of unbalanced mass to the axis of rotation

of the drive shaft; ω – the angular velocity of the blade shaft.

The torque is:

$$M_k = F_k \cdot a = m_1 \cdot L_d \cdot \omega^2 \cdot a = m_1 \cdot L_d \cdot \dot{\varphi}_1^2 \cdot a \qquad (2)$$

where a – the distance between the lines of action of forces F_k, φ_1 – the tilt angle of unbalanced masses m_d.

The developed system has 4 main masses and 3 degrees of freedom. The main masses of the system are the masses of unstable elements or unbalanced masses m_1, the drive shaft and bearing units on which it stands – m_2, the propeller or screw blades and the mounting unit – m_3, the scraper with the mounting unit and the brush or rubber elements for cleaning the bottom of the hydrolysers **(Bandura et al., 2015; Palamarchuk et al., 2016; Couzin et al., 2005).**

The basic degrees of freedom of the system include the angle of rotation of the unbalanced masses φ_1, the linear displacement of the drive shaft X_2, the angular displacement of the drive shaft ψ_2.

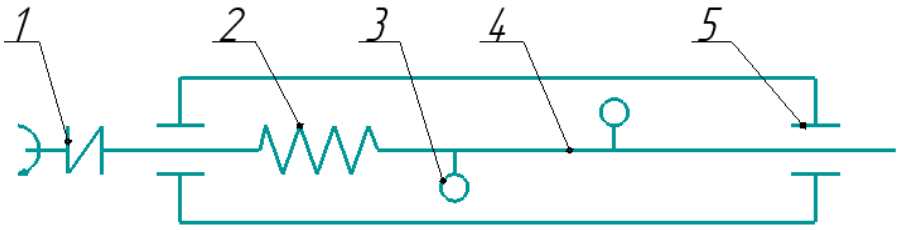

Figure 1 Model of mechanical unbalanced vibration excitation of the oscillatory motion of the blade shaft of a hydrolyser of pectin-containing raw materials: 1 – spring coupling, 2 – spring element, 3 – unstable element in the form of unbalanced masses, 4 – blade shaft, 5 – shaft bearing.

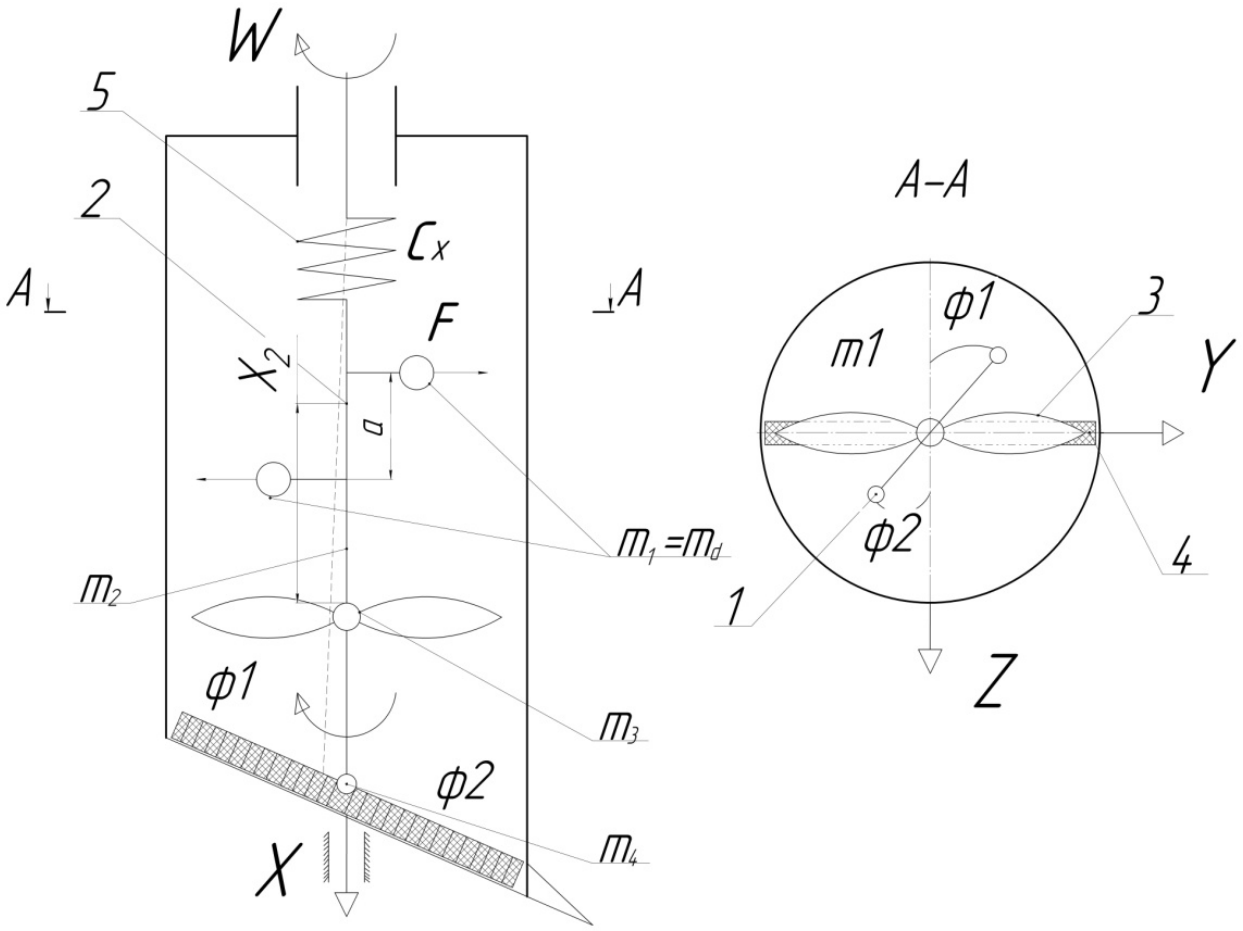

Figure 2 Calculation model of pectin-containing raw material hydrolysers with vibrating centrifugal mixer: 1 – unbalanced masses, 2 – drive blade shaft, 3 – blade, 4 – scraper, 5 - spring element with rigidity C_x.

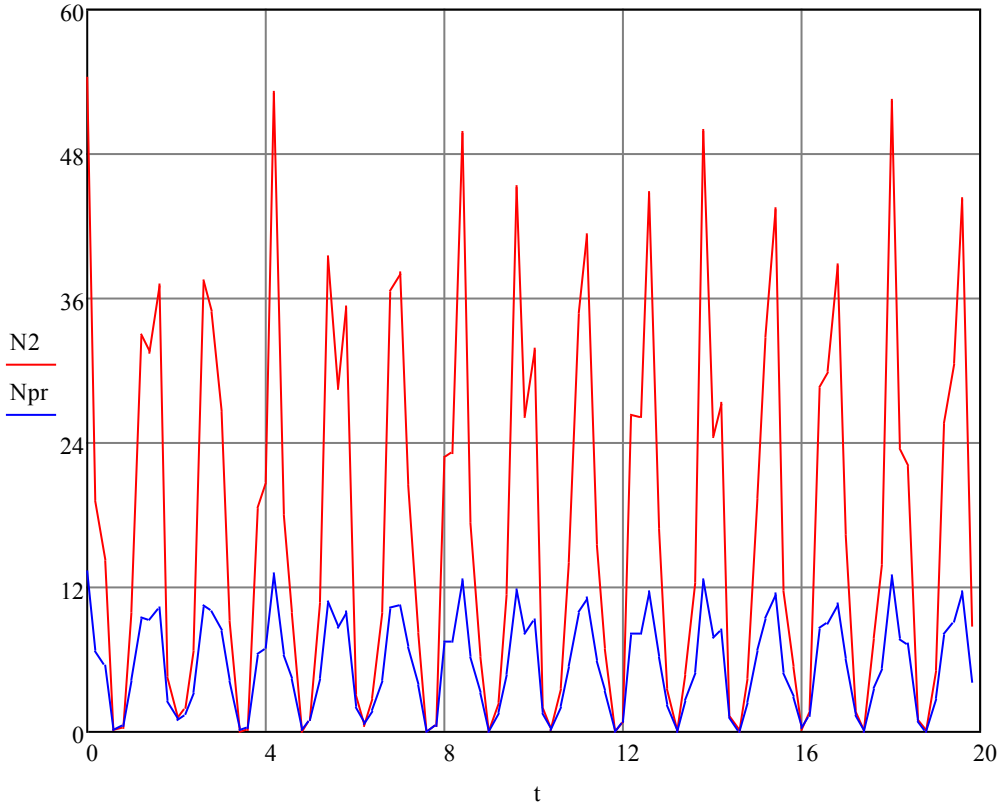

Figure 3. Energy (a) and power (b) characteristics of the system under study versus the processing time t: N_2 – power on the drive shaft of the agitator · 10^2W; N_{pr} – the power consumption due to the resistance of the technological environment ·10^2 W; F_2 - the driving force of the process ·10^2 N; P_r is the resistance of the technological environment · 10^2 N.

The Lagrange method is effective when composing the differential equations of motion of the working elements of the system, representing the desired expressions in the following system of equations:

$$\begin{cases} \left(\ddot{X}_2\right)+K_X^2\cdot X_2=K_m\cdot L_d\cdot(\dot{\varphi}_1)^2\cdot\left(1-f_{fr}\right) \\ \left(\ddot{\varphi}_1\right)+\left[m_1\cdot L_d\cdot a\cdot f_{fr}\right]\cdot(\dot{\varphi}_1)^2= \\ =\left[m_1\cdot L_d\cdot\dfrac{g}{I_0}\right]\cdot\sin\varphi_1+\dfrac{\left[M_{res}-P_r\cdot r_b-M_{ad}\right]}{I_0} \\ \left(\ddot{\varphi}_2\right)+K_\psi^2\cdot\psi_2=\left(I_2+I_3+I_4\right)^{-1}\cdot \\ \cdot\left(m_2\cdot g\cdot r_в+m_1\cdot L_d\cdot a\cdot(\dot{\varphi}_1)^2\right) \end{cases} \quad (3)$$

where: k_m – mass utilization coefficient; k_x^2 , k_φ^2 , k_ψ^2 – natural oscillation frequencies; C_x ,C_φ ,C_ψ – the rigidity of the spring element relative to the free motion of the coordinate; g –the gravitational acceleration; $r_в$ – the shaft radius, P_r – the fluid resistance forces; f_{fr} – the friction coefficient; r_b – the blade radius; M_{res} – the moment of resistance; M_{ad} – the moment of resistance by adhesive forces; adhesion I_0 , I_1 , I_2 , I_3 , I_4 – the moments of inertia of the corresponding masses of the system under study.

For the composed system of equations (2), taking into account the practical aspects of the implementation of the process under study, the following assumptions can be used: given the sufficiently small angular displacement of the drive shaft, it can be neglected, i.e. ψ = 0; we assume that the drive shaft rotates at a constant angular velocity, i.e. ω = const; the adhesive forces of the sediment to the bottom can be neglected, i.e. $M_{зч}$=0.

The system of equations for the independent constants φ_1 and x_2 was solved using the Cauchy method, in particular, for the first two expressions were obtained:

$$X_2=\frac{A_x}{K_X^2\cdot\left(1-\cos K_X t\right)}= \\ =\left[\frac{m_1\cdot\left(1-\cos K_X t\right)\cdot L_d\cdot\omega^2\cdot\left(1-f\right)}{K_X^2\cdot\left(m_2+m_3+m_4\right)}\right] \quad (4)$$

$$\varphi_1=m_1\cdot L_d\cdot g\cdot\frac{\left(\sin\omega t-\left(\dfrac{\omega}{K_\varphi}\right)\cdot\sin K_\varphi t\right)}{I_0\cdot\left(K_\varphi^2-\omega^2\right)}+ \\ +\left[\frac{\left(M_{Kp}-P_r\cdot r_л-m_1\cdot L_d\cdot a\cdot f_{sl}\cdot\omega\right)\cdot\left(1-\cos K_\varphi t\right)}{I_0\cdot K_\varphi^2}\right] \quad (5)$$

where: A_x – coefficient that was introduced to simplify the expression $A_x=K_m\cdot L_d\cdot\omega^2\cdot\left(1-f\right)$, then $\ddot{X}_2+K_X^2+X^2=A_X$, t – the operating time of the corresponding element; f_{sl} – the slipping coefficient.

Resistance, created by the fluid when the mixer's working elements rotate in it, without friction is:

$$P_r=K_0\cdot K_{OП}\cdot S_0\cdot\left(v_b\pm v_v\right),H \quad (6)$$

where: K_o – the experimental coefficient; K_{op} – the optical coefficient; S_o – the area of the projection of the working element to a plane perpendicular to the velocity vector of the blade, m^2; υ_b - the blade velocity; υ_v – the velocity of the liquid.

Based on the obtained dependences (4, 5, 6) and using the MathCAD, we created the following graphs of the power and energy characteristics of the system under study, depending on the processing time t in Figure 3 and the angular velocity of the blade shaft ω in Figure 4 and Figure 5.

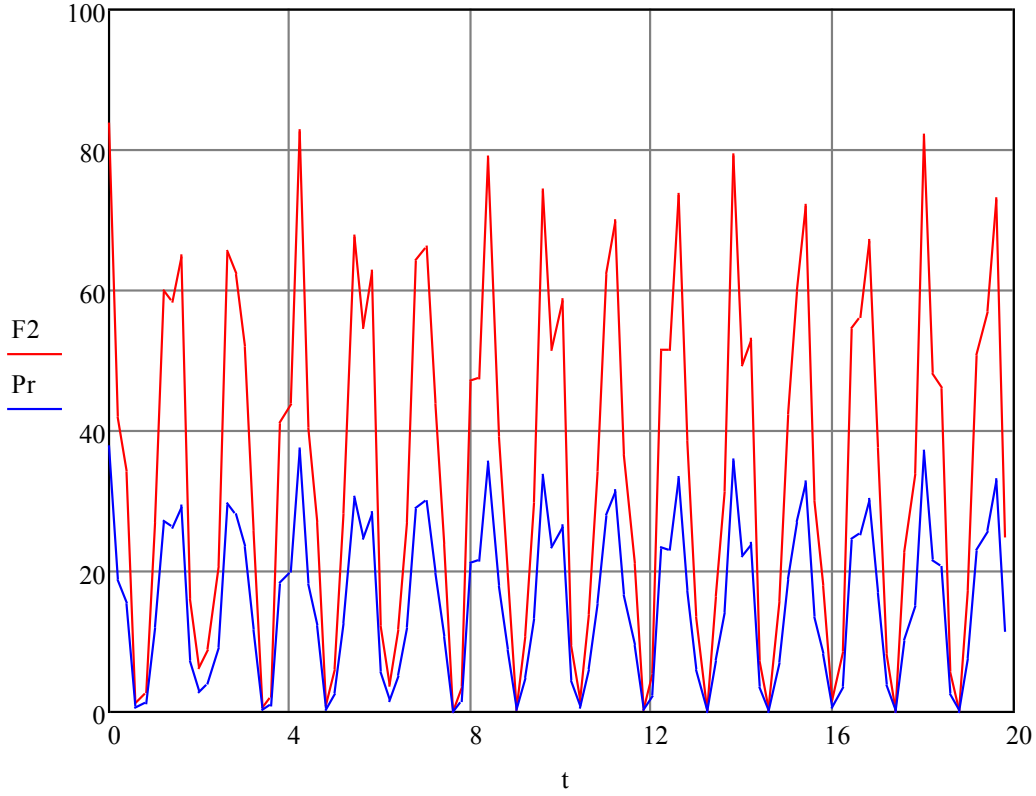

Figure 4. Energy characteristics of the system under the study N_2 and N_{pr} ($\cdot 10^2$ W) versus the angular velocity of the blade shaft ω ($\cdot 10^2$ rad/s).

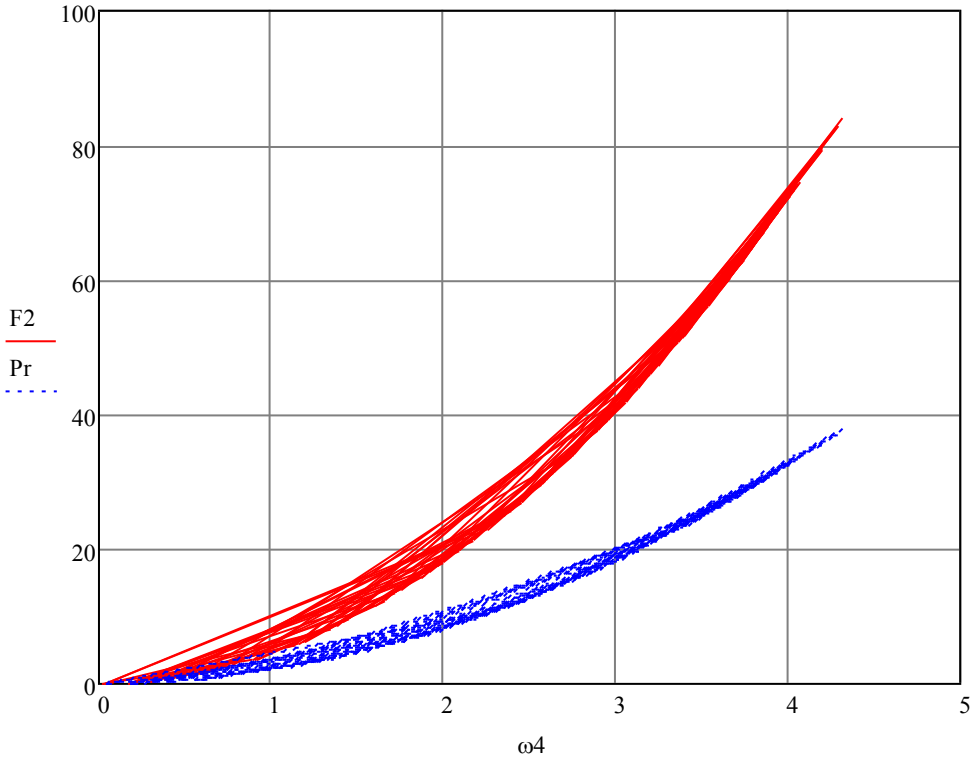

Figure 5. Force characteristics of the system under the study F_2 and P_r ($\cdot 10^2$ N) versus the angular velocity of the blade shaft ω ($\cdot 10^2$ rad/s).

Table 1 Different methods of the hydrolysis process of crushed pumpkins.

Suspension mixing conditions	Time of hydrolysis, s	Dissolved pectin content, %	Jelly-forming capacity of paste, mmHg
Without vibration, the angular speed of the stirrer shaft is ω = 150 rad/s	3600	1.5	315
With superimposing of 500 W of the vibration power using the same angular velocity	2400	2.8	378

From Figure 3 it is obvious that in the implementation of the experimental hydrolysis process with vibration centrifugal activation, the driving force is sufficient to overcome the resistance of the liquid technological environment. Also, 2.3 – 2.5 kN of driving force and 2.4 – 3.0 kW of drive motor power can be used to intensify this process **(Zavialov et al., 2015; Yanovich et al., 2015; Chuang et al., 2007; Carrillo et al., 2007)**.

For the angular velocity $\omega = 150$ rad/s of the drive shaft, the power consumption is 600W with a resistance of the fluid environment of 250W, and, if the angular velocity is doubled, the energy consumption increases by 7.2 times in Figuru 4. There is a significant increase in the technological impact of the vibration centrifugal driving force of the experimental hydrolysis process - from 1.5 kN for $\omega = 150$ rad/s to 4.6 kN for $\omega = 300$ rad/s sn Figure 5, which requires a corresponding significant energy consumption of 2.4 kW in Figure 4. Therefore, the effective angular velocity of the drive mechanism of the designed hydrolysers can be considered $\omega = 100 - 150$ rad/s, which is also recommended to provide the necessary reliability parameters of such equipment.

Pectin paste from the pumpkin "Hybrid – 75" was made on the developed hydrolysers to experimentally confirm the positive effect of hydrolysis of plant raw materials in the conditions of vibration mixing of the hydrolysis suspension.

The hydrolysis of the crushed pumpkin was performed at a temperature of 80 – 85 °C in the apparatus under a pressure of 0.05 – 0.1 MPa with 1.5 % solution of lactic acid **(UIIP, 1993)**, in the hydromodule GM 15, with the temperature of the hydrolyzed suspension during the experiment is not exceeded 65 °. After the addition of sugar syrup with sucrose content of 41% was carried out by evaporation at a temperature of heating medium 90 – 92 °C in the apparatus at a pressure of 0.1 – 0.15 MPa for 60 – 70 min. to a dry matter concentration of 68 – 70 %, with the temperature of the hydrolyzed suspension during the experiment did not exceed 70 °C. Hydrolysis was performed by mechanical stirring without the use of vibrational vibrations of the system and after vibration excitation of the shaft of the hydrolyzer.

The content of oxymethylfurfural in the finished product was determined in Ukrainian Laboratory for Quality and Safety of Agroindustrial complex according to state standard of Ukraine 7466-2001 **(DSTU 7466-2001)**. For which the permissible content did not exceed 25 mg / kg.

The standard complies with ISO 7466: 1986 Fruit and vegetable products - Determination of 5-hydroxymethylfurfural (5-HMF) content.

The results of the experiments are given in Table 1.

The Table 1 shows that the vibrational effect on the hydrolysis suspension allows to reduce the hydrolysis time by 1.5 times, increase the amount of dissolved pectin of higher quality by the jelly-forming capacity in the paste by almost 2 times.

CONCLUSION

Based on the analysis of the activation methods of technological flows during the implementation of the experimental hydrolysis process, a vibration centrifugal scheme of its intensification was selected, which potentially allows reducing the energy consumption for mixing of pectin-containing raw materials of plant suspension; increasing the driving force of the process, increasing the degree of mixing; improving the conditions of cleaning of the bottom of the hydrolysers from the sediment; and carrying out the self-cleaning of working elements.

Based on the mathematical model, analytical and graphical dependencies were obtained for the main power and energy characteristics of the process, which confirmed the overcoming of technological resistance of the liquid environment in the entire speed range of the drive shaft with the potential for process intensification at the power consumption of 2.4 – 3.0 kW, or a driving force of 2.3 – 2.5 kN.

The effective mode of operation of the drive mechanism of the designed hydrolysers is recommended: the angular speed of the shaft is $\omega = 150 - 150$ rad/s, which requires power consumption of 500 – 600 W.

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EFFECT OF PROCESS PARAMETERS ON THE FUNCTIONAL AND PHYSICOCHEMICAL PROPERTIES OF EXTRUDATES ENRICHED WITH STARCH-BASED NUT FLOUR

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ABSTRACT
Widening the range of products produced on the basis of agricultural raw materials and improving the quality of these products and increasing their nutritional value represent urgent challenges. Therefore, the production of new mass consumption products with high nutritional and biological value brings to the fore the use of local nut flour as an enriching supplement in innovative technological processes. The high nutritional value of nuts (nuts, walnuts, and peanuts) is due to their chemical composition, including lipids, a large amount of soluble proteins that are well absorbed by the human body, sufficiently large quantities of vitamin B1 and a small amount of vitamins PP and E. It is known that in peanut grains, lipids have a balanced composition of fats and acids, as well as sufficiently large amounts of essential amino acids, which makes their protein composition closer to that of animal proteins. This study considers the influence of thermoplastic extrusion parameters on the functional and physicochemical properties of extrudates in their formation process. The technological and design parameters of the process and their variation ranges are based on studies conducted on model systems. The ratio of the extrusion mixture components (formulation) is also developed. Based on the methodology for multifactorial experimental design, the variation of the volume weights, expansion rates, and mechanical specific energy expenditure of porous extrudates enriched with starch-based nut flour is studied. It has been established that the best quality indicators of the products are achieved with the minimum volume weight and the maximum expansion rate.

Keywords: extrudate; process; starch; walnut; parameter

INTRODUCTION
Extruded products with different compositions and functional properties are produced on the basis of starch-containing raw materials using various plant and animal supplements. We have experimentally substantiated the use of non-traditional raw materials, namely, nut flours, in the production of porous extrudates, as enriching supplements. This will expand not only the range of these products, but will also improve their quality and nutritional value. **(Sesikashvili, Zverev and Berulava, 2018)**.

Based on previous studies, we have investigated the influence of raw materials in shaping the structure of extrudates and conducted studies on extrudate model systems, using starch gel from various origins. This article describes the process of producing extrudates by the method of thermoplastic extrusion as a thermotropic process for the formation of biopolymer gels in the stream **(Tsagareishvili et al., 2019)**. The study of the properties of the obtained gels allows us to identify the moisture function in the process of thermoplastic extrusion, as well as during the storage of the obtained product. It is established that:

- During the process of extrusion, moisture determines the gelatinization point of the processed raw materials, whilst also influencing the shaping of the structure of extrudates **(Tsagareishvili et al., 2019)**.
- Minimal physicochemical transformations occur in gels in an amorphous state, that is, when their moisture content is minimal. Therefore, the storage of porous extrudates is recommended at low (4–7%) moisture contents **(Tsagareishvili et al., 2019)**.

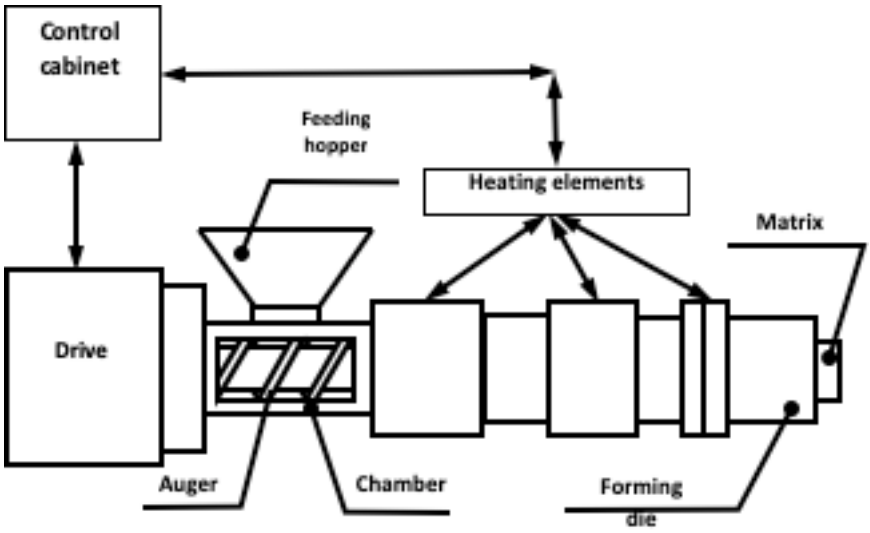

Figure 1 Schematic of the extruder.

Based on a literature review, it can be concluded that in addition to the composition (formulation) and technological parameters (moisture content, temperature and processing time), the shaping of the structure of the extrudates is also influenced by the parameters of the thermoplastic extrusion process (technological and design parameters).

Currently, the development of new formulations for products manufactured by the method of thermoplastic extrusion is mostly based on the empirical selection of the components of processed raw materials and the process operating and design parameters. In the best-case scenario, the systems analysis method is used for the multifactorial process **(Van Lengerich, Meuser and Pfaller, 1989; Meuser, 1984)**.

(Meuser, 1984) showed that the functional properties of the product depend on the system parameters of the process, with the latter being a function of the process parameters (moisture content, temperature, auger speed, die hole diameter and pressure on the auger).

Therefore, the task of studying the structure of extrudates is based on studying the forms of the isotropic and anisotropic microstructural forms of products based on starch, proteins or their mixtures. As shown in the thermoplastic extrusion process scheme (Figure 1), the raw materials to be processed in the mixing zone are moistened, mixed and by means of the auger, they move into the channel and in the melt zone transform into a plastic state. Studies by **(Van Lengerich, Meuser and Pfaller, 1989; Meuser, 1984)** note that during the process of biopolymer flow movement, under the action of shear deformation at the end of the cylinder and in the forming die, there occurs the process of especially intensive structuring of the melt. After leaving the forming die, the product is finally formed.

In order to explain the mechanism for shaping the anisotropic structure of extrudates **(Harper, 1986)**, a concept was proposed where macromolecules change their position under the action of shear deformation when raw materials transform into a plastic state during the gelatinization of starch and the denaturation of proteins. During the melt cooling process, the macromolecules bind and link together.

Based on the above, the purpose of this study is to investigate the influence of the extrusion process parameters on the functional characteristics of the extrudates (volume weight – ρ and expansion rate - Exp) and physicochemical properties (mechanical specific energy - E).

SCIENTIFIC HYPOTHESIS

Based on the studies conducted, the influence of thermoplastic extrusion process parameters (W, T, n, s and d) on the formation of the functional (ρ and Exp) and physicochemical parameters (E) of extrudates will be determined. The advantage of using the systems analysis method for solving the optimization problem to produce functional products with useful properties will be shown.

MATERIAL AND METHODOLOGY

For the purpose of carrying out experimental studies, in accordance with the formulations, we selected the following materials: cornflakes – **GOST 6002-69**; corn starch - **GOST 7697-82**; walnut, peanut and table salt - **GOST 13830-84**.

For the extrudates, we used an extruder K - 30 (Ukraine), composed of an extrusion chamber, an auger kit, a forming die with different matrix diameters and a control panel.

The extruder chamber is a hollow cylinder with a 400 mm length and a 19 mm inner diameter of the auger, with six longitudinal channels designed to transport the mass processed when using raw materials with the floury structure. On the outer surface of the cylinder, there are two mounted heating elements. In general, the extruder has three zones: mixing, plasticizing and charging. From the top of the cylinder, there is a vertical single-screw proportioning feeder secured with a pyramid hopper. Inside the chamber, a single-thread variable-pitch auger is placed with an outer diameter of 19 mm.

Table 1 Factor variation range.

	Factors	Levels				
#		1	2	3	4	5
1	Mixture's moisture content, %	15	20	25	30	35
2	Temperature in the cylinder, °C	150	160	170	180	190
3	Auger speed, min^{-1}	150	170	190	210	230
4	Auger charging degree	1	2	3	4	5
5	Matrix diameter, mm	2	3	4	5	6

Table 2 Experiment performance grid.

#	W%	T °C	n min^{-1}	S	d
1	15	150	150	1	2
2	15	160	170	2	4
3	15	170	190	3	3
4	15	180	210	4	6
5	15	190	230	5	5
6	20	150	170	4	4
7	20	160	230	1	3
8	20	170	210	3	6
9	20	180	150	2	5
10	20	190	190	5	2
11	25	150	190	2	6
12	25	160	170	5	5
13	25	170	230	4	2
14	25	180	210	1	4
15	25	190	150	3	3
16	30	150	210	5	3
17	30	160	150	4	6
18	30	170	190	1	5
19	30	180	170	3	2
20	30	190	230	2	4
21	35	150	230	3	5
22	35	160	210	2	2
23	35	170	150	5	4
24	35	180	190	4	3
25	35	190	170	1	6

During the studies, we used the auger kit with varying values of charging. At the end of the extruder chamber, the forming die with a matrix is connected by a threaded connection.

We used matrices with different hole diameters. We varied the auger speed from 150 to 230 min^{-1} and measured the rotary speed by means of a tachometer. The temperature in the cylinder was measured using a thermocouple.

The testing methodology used in the experiments is as follows. In the preliminary studies, the ratio of the extrusion mixture components was determined by a sensory analysis of taste, color and rigidity:

- Cornflakes – 56.8%;
- Corn starch – 10%;
- Walnut – 12%;
- Peanut – 4.5%;
- Table salt – 0.7%;
- Moisture content of mixture with added water – 16%.

During the experiments, the moisture content of the mixture varied between 16% and 35%. The mixture components were hydrated before the extrusion process and settled at a temperature of 5 °C for 24 h. We extruded the mixture for the determined values of moisture content, temperature, auger speed, auger types and matrix size. A temperature of 70 °C was maintained in the feed zone of the extruder.

After the extruder was operated in a stable mode, we took samples and determined their volume weights and expansion rates, and in parallel, we calculated the mechanical specific energy expenditure.

The volume weights of the extrudates were determined using vessels of a previously-known capacity of $0.5 \cdot 10^{-3}$ dm^3 that were filled with extrudates and then weighed on an analytical balance. The volume weight was calculated by the following formula (1):

$$\rho = G / V \quad (1)$$

where G is the weight of the extrudates, kg, and V is the volume occupied by the extrudates, m^3.

The expansion rate of the extrudates was calculated as follows (2):

$$Exp = D / d \quad (2)$$

where D is the extrudate diameter, mm, and d is the matrix die hole diameter, mm.

The mechanical specific energy was calculated using the formula (3):

$$E = (M \cdot n) / Q \quad (3)$$

where M is the auger torque, nm, N is the auger speed, min^{-1}, and Q is the extruder capacity, kg/h.

To further optimize the process of obtaining the base product, we used a mathematical method multifactorial experimental design **(Grachev, 1979)**. The main factors for obtaining the base product were the moisture content of the extrudate mixture (W), the temperature in the forming die of the extruder (T), the auger's number of rotations (n), the charging degree of the extrudate (S) and the matrix diameter (d). According to **(Grachev, 1979)**, in order to further optimize the process in the case of five-factorial experiments, it is necessary to conduct 25 experiments and vary each factor at five levels. The factor variation range was chosen on the basis of literature reviews and studies that we conducted previously on model systems. The factor variation range is given in Table 1.

The experiment performance grid is shown in Table 2.

STATISTICAL ANALYSIS

To analyze the test parameters (the moisture content of the starch paste, gelatinization point, starch paste transparency, starch paste embrittlement temperature and starch paste modulus of elasticity) of the extrusion products, a statistical analysis of the data was carried out and the reliability of the data was evaluated by a T-test using the Windows IBM SPSS Statistics program (version 20.0). To describe the ordered sample, we used the statistical functions of the average arithmetic value and the average standard error. A graphical interpretation of the results was made using Microsoft Excel. Figure 2, Figure 3 and Figure 4 illustrate the data of typical tests and each value is an average of at least ten determinations. We selected the value of reliability as $p < 0.05$.

RESULTS AND DISCUSSION

As is known, any production technology requires establishing the process parameters. The extrusion process parameters are as follows: the moisture content of raw materials, the temperature in the extruder's cylinder, the auger speed, and the extruder design parameters (the auger type and a matrix die hole diameter).

Based on the experimental studies, we have determined the relationship between the process parameters and the functional and physical characteristics of extrudates. The results of the studies are presented in Table 3.

Based on the experimental results, we have constructed the experimental curves for the relationship between the process parameters and the functional and physical characteristics of extrudates.

Figure 2 illustrates the influence of the process parameters on the volume weight of extrudates. As the graphs show, the moisture content of the mixture has a particular influence on the volume weight. The best indicator of this functional characteristic is in the 15-20% moisture content range. Further increase in the moisture content results in an increase in the volume weight, which worsens the quality of extrudates - the extrudate becomes more rigid. All other parameters in the same moisture content range do not adversely affect the volume weight variation. The end product is more airy and tender.

Enrichment of rice-based extrudates with the salts of calcium reduces the volume weight from 0.224 to 0.126 $g \cdot cm^{-3}$, and the expansion index from 3.21 to 2.93 (**Janve and Singhal, 2018)**. Sugar crystallization during the thermo-hydro-mechanical processing of extrudates based on corn starch with sugar-containing supplements reduces the degree of expansion of extrudate **(Wang, Gu and Ganjyal, 2019)**. Infusion of nitrogen injection during extrusion significantly increases the degree of expansion of extrudate and its porous structure **(Li, Masatcioglu and Koksela, 2019)**.

Table 3 The influence of the process parameters on the functional and physical characteristics of extrudates

	Functions	Levels				
#		1	2	3	4	5
1	ρ (W) 10^{-3}	0.160	0.144	0.225	0.311	0.360
2	ρ (T) 10^{-3}	0.234	0.214	0.218	0.234	0.300
3	ρ (n) 10^{-3}	0.222	0.236	0.260	0.272	0.210
4	ρ (S) 10^{-3}	0.262	0.244	0.210	0.234	0.250
5	ρ (d) 10^{-3}	0.228	0.218	0.241	0.245	0.268
6	Exp (W)	1.72	2.52	2.2	2.0	1.56
7	Exp (T)	1.68	1.88	1.92	2.01	2.51
8	Exp (n)	1.95	1.78	1.85	1.95	2.47
9	Exp (S)	1.34	1.96	2.14	2.22	2.34
10	Exp (d)	2.25	2.43	2.11	1.90	1.31
11	E(W) 10^{-3}	7.725	7.197	6.801	6.450	4.332
12	E(T) 10^{-3}	7.235	7.568	6.930	6.720	4.052
13	E(n) 10^{-3}	4.950	5.212	6.803	7.015	8.525
14	E(S) 10^{-3}	4.514	5.520	6.529	7.502	8.440
15	E(d) 10^{-3}	8.110	8.052	7.603	5.224	3.516

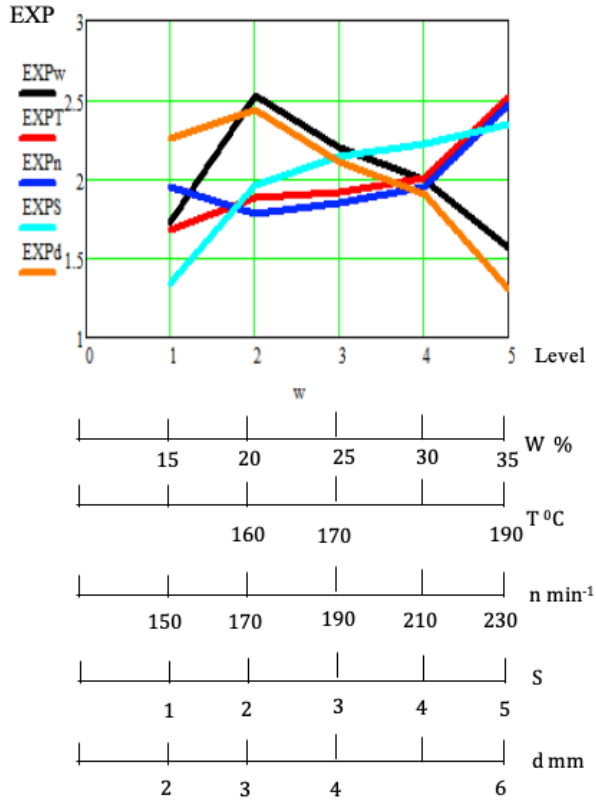

Figure 2 The influence of the process parameters on the volume weight of extrudates.

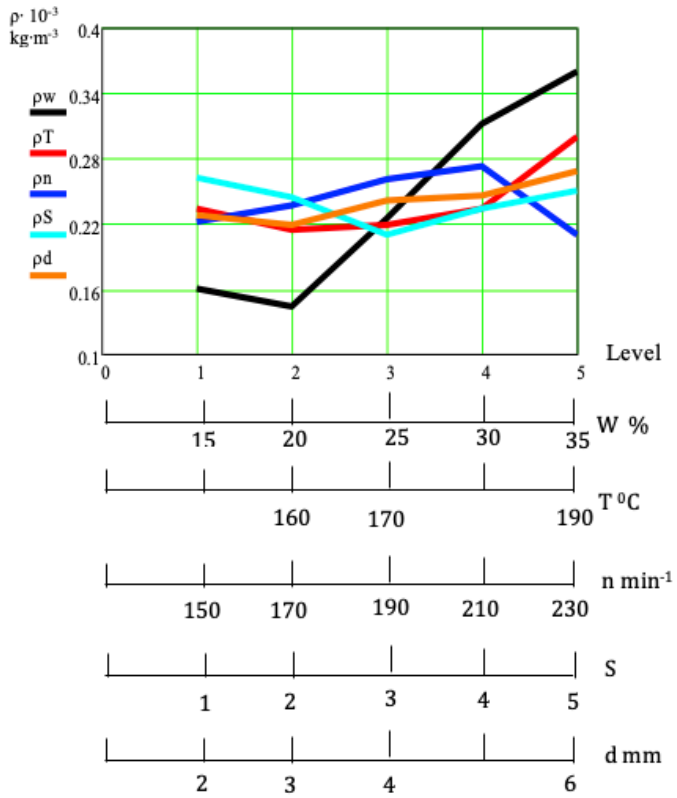

Figure 3 The influence of the process parameters on the expansion rate of extrudates.

The addition of coconut to extrudates produced from corn and rice flour reduces the degree of expansion, increases the volume weight and is characterized by relatively dark color, but they were rich in protein, minerals, and have antioxidant properties **(Arivalagan et al., 2018)**.

Many studies **(Skamniotis et al., 2018; Beck et al., 2018; Do Carmo et al., 2019; Da Silva Teba et al., 2017; Singh et al., 2019)** have reported similar functional characteristics of extrudates enriched with various supplements and their results concur with ours.

Figure 3 illustrates the influence of the process parameters on the expansion rate of the extrudates.

It is known that the greater the expansion rate of the extrudates, the greater the number of porous masses and the better their visual side and organoleptic characteristics. Compared to other parameters, the moisture content of the raw materials and the matrix die hole diameter have a greater influence on the expansion rate of the extruder. The maximum expansion rate is observed in the case of a 20% moisture content and a matrix die hole diameter of 3 mm.

In this case, the relatively low values of the volume weights and expansion rates of the extrudates, compared to the similar data of extrudates based on pure starch or corn flour, is explained by the supplement of nut flour, due to the fat content, which partially impedes the extrusion process.

Enrichment of extrudates based on corn flour with barley, fondant apple and sugar-beet reduces the degree of their expansion, deteriorates their structure, increases their density and firmness, while addition of 1% pectin improves the degree of their expansion **(Ačkar et al., 2018)**. When studying the physical properties of extrudates produced corn and yellow chickpea, it has been established that the best results were achieved by thermoplastic extrusion process parameters as follows: cell temperature - 158.64 °C, auger speed -372 m^{-1}, moisture content -18.4% **(Jacques-Fajardo et al., 2017)**. Regression analysis of the production of functional extrudates with high nutritional value based on corn flour by adding of dry broccoli or olive paste extrudates revealed that the increase in the concentration of broccoli or olive paste, as well as reducing the temperature and auger speed led to obtaining more dense extrudates with lower density.

At a moisture content of 14% and a 4%-concentration of product and the, extrudates had the highest expansion ratio at an auger speed of 250 m^{-1} **(Bisharat et al., 2013)**.

The relationship between the expansion rate of extrudates and the process parameters has been extensively studied **(Arivalagan et al., 2018; Sharma, Srivastava and Saxena, 2019; Carvalho et al., 2010; Zhang et al., 2020; Wang, Gu and Ganjyal, 2019)**. These studies confirm the accuracy of the tests that we conducted.

Figure 4 illustrates the influence of the thermoplastic extrusion process parameters on the mechanical specific energy. The plots clearly show the influence of each process parameter on mechanical specific energy expenditure, in particular, the increase in the rotary speed and in the charging rate of extruder results in increasing the mechanical specific energy, while the increase in the moisture content, temperature and matrix die hole diameter leads to a downward trend in the share of the mechanical specific energy. The results obtained concur with the previous studies **(Jacques-Fajardo et al., 2017; Sharma, Srivastava and Saxena, 2019; Carvalho et al., 2010; Beck et al., 2018)**.

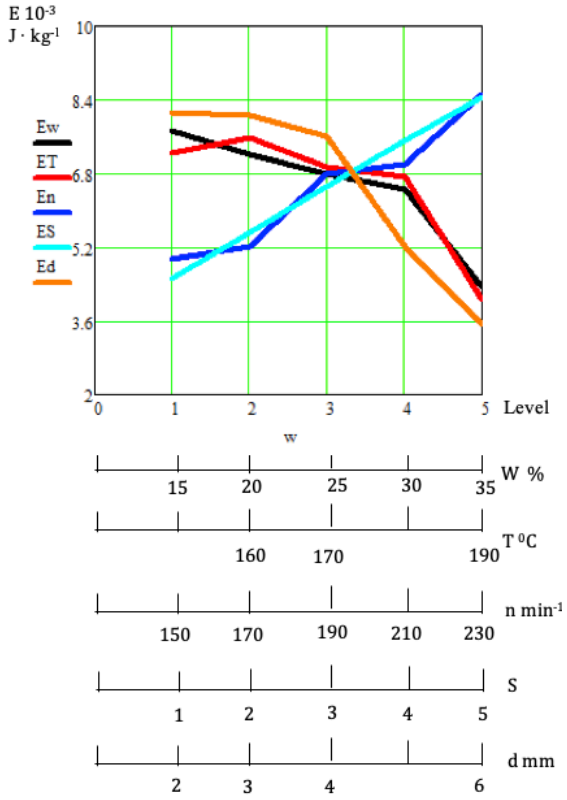

Figure 4 The influence of the thermoplastic extrusion process parameters on the mechanical specific energy.

CONCLUSION

This work allows us to understand the physical nature of the influence of each factor affecting the thermoplastic extrusion process on the functional and physical characteristics of extrudates enriched with nut flour.

By varying the process parameters, studies of the volume weights and the expansion rates show that they are inversely proportional to each other. In particular, the greater the extrudates expansion rates, the less their volume weights. In addition, the obtained products are airier, more tender and have better nutritional and consumer values. Therefore, in order to optimize the process of producing extrudates with a porous structure, it is necessary to solve the optimization problem: ρ(W; T; n; s; d) = min; Exp(W; T; n; s; d) = max.

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^{137}Cs ACTIVITY CONCENTRATION IN MUSHROOMS FROM THE BOBRŮVKA RIVER VALLEY

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ABSTRACT
A total of 505 mushrooms belonging to 9 species were collected in the 2017 and 2018 mushrooming seasons near Dolní Rožínka in the Bohemian-Moravian Highlands and analysed by gamma spectrometry for ^{137}Cs activity. The greatest ^{137}Cs activity of 575 Bq kg^{-1} was detected in the species *Boletus edulis*, which is just below the permitted limit in its native state. In contrast, the detected activity level was only 316 Bq kg^{-1} in the mushroom *Imleria badia*, which is reported to be associated with the highest cumulative capability of all fungi species. However, differences in mean contamination values were not significant (p <0.05) due to high variability. It was shown that activity concentration is not dependent on the weight (size) of *Imleria badia*. Our results also confirmed the generally well known lower ^{137}Cs activity in *Russula* species belonging to the group of gill-bearing or lamella-bearing mushrooms.

Keywords: radiocaesium; mushrooms; river valley; *Boletus*; *Imleria*

INTRODUCTION
The ^{137}Cs isotope is one of more than 20 radioactive isotopes of caesium. ^{137}Cs isotopes are formed at relatively high concentrations, reaching as much as 6.3 %, during the fission (nuclear chain) reaction in nuclear reactors **(Söderlund et al., 2011)**.

Its negative biological effect is due to the emission of beta particles with the subsequent conversion of ^{137}Cs to metastable ^{137}Ba, which emits gamma photons of an energy of 661.7 keV **(Wasek et al., 2014)**.

Despite the fact that more time than the physical half-life of ^{137}Cs (30.17 years) has elapsed since the Chernobyl nuclear power plant accident (26 April 1986), there are still geographical areas with long-term persistent contamination **(Smith and Beresford, 2005; Beňová et al., 2016)**.

There are species of mushrooms in coniferous forests with a great ability to accumulate radiocaesium. Mushrooms probably contribute to the long-term retention of radiocaesium in organic layers of forest soils, especially spruce stands **(Steiner, Linkov and Yoshida, 2000)**. The radiocaesium activity concentration in mushrooms from different areas of the Czech and Slovak Republics in 2000–2004 was reported by **Dvořák et al. (2006)**.

The highest ^{137}Cs activity concentration, representing 6.263 Bq kg^{-1} of dry matter, was measured in *Imleria badia* (syn. *Xerocomus badius*) from the area called Staré Ransko (the Bohemian-Moravian Highlands in the Czech Republic). The results also show significantly lower levels of ^{137}Cs activity in Slovakia as compared to the Czech Republic.

^{137}Cs activity depends not only on the contamination of the environment, but also on the mushroom species **(Kalač, 2001)**. The highest levels of radioactive caesium contamination are found in saprophytic mushrooms **(Duff and Ramsey, 2008)**. The mycelium of the non-edible mushroom *Lactarius turpis* (formerly *L. necator*) in Ukraine (1996 – 1998) showed an average activity of 52.700 Bq kg^{-1} **(Vinichuk and Johanson, 2003)**.

Most of the published papers are based on mushroom collection from a relatively large area **(Chiaravalle et al., 2018)**. In such large areas there are a number of different factors that affect the final mushroom contamination **(Kalač, 2001; Dvořák et al., 2006)**.

First and foremost, the deposition of ^{137}Cs is unevenly distributed depending on the precipitation intensity, latitude and altitude of the area **(Lehto, Vaaramaa and Leskinen, 2013; Bulko et al., 2014)**. The contamination of Europe by post-Chernobyl radiocaesium shows a mosaic pattern with significant differences in relation to area activity **(Nilsson, 2009)**.

Scientific hypothesis

The aim of this work is to compare the ^{137}Cs contamination of various mushrooms in a small relatively homogeneous conifer forest ecosystem in a river valley.

MATERIAL AND METHODOLOGY

Mushrooms were collected at a defined location in the Bobrůvka River Valley (near Dolní Rožínka in the Bohemian-Moravian Highlands) in the 2017 and 2018 mushrooming seasons. A total of 505 mushrooms of 9 different species, both common and rare in this particular area, were collected **(Table 1)**.

Native mushroom samples were homogenised. Activity determination was carried out in the geometry of a 450ml Marinelli vessel or a 200ml polyethylene vial. These two geometries corresponded to the quantities of mushrooms per sample.

Determination of ^{137}Cs activity was performed by two gamma spectrometry systems using HPGe GC4018 (40% efficiency) and HPGe GC2020 (20% efficiency) germanium detectors, both with a resolution of 1.8 keV, verified by the Czech Metrology Institute. The measurement time was 18 hours.

The programs Genie 2000 (Canberra) and Gamwin (Nuwia Třebíč) were used for evaluation. The ^{137}Cs activity in all measured samples was higher than the minimum detectable activities (MDA). In addition to the mass activity, the total combined standard uncertainty was calculated using the formula:

$$u_a = \left(u_E^2 + u_p^2 + u_y^2 + u_s^2 + u_{ss}^2 + u_r^2 + u_t^2 + u_A^2 + u_M^2 \right)^{\frac{1}{2}}$$

where the individual standard uncertainties are as follows: relative uncertainty for efficiency (uE), relative uncertainty for peak area (up), relative uncertainty for yield (uy), relative uncertainty for time coincidence summing (us), relative uncertainty for electronic stability (uss), relative uncertainty for time (ut), relative uncertainty for decay (ur), relative uncertainty for self-absorption (uA), relative uncertainty for reproducibility (uM).

Statistical analysis

The weighted arithmetic mean, average total standard uncertainty, and maximal and minimal value of mass activity were calculated for statistical evaluation. A two-sample unequal variances t-test was used to compare the mean differences. A correlation coefficient was calculated to compare the dependencies.

RESULTS AND DISCUSSION

Mushrooms, as one of the most important components of the forest ecosystem, are able to accumulate a significant amount of radionuclides, including ^{137}Cs **(Heinrich, 1992; Škrkal et al., 2013; Guillen and Beaza, 2014)**. The first mention of contamination of mushrooms with radiocaesium comes from the nineteen sixties. Mass activity values in a range of 10 to 2.500 Bq kg^{-1} have been published in Germany (Grüter, 1964 and 1971). According to **Kalač (2001)**, contamination comes from nuclear weapons tests. Other factors that can affect the value of mushroom contamination in addition to the growth medium properties are the climate and the associated amount of precipitation, as well as the sampling time and the fungus type itself **(Heinrich, 1993)**. Furthermore, it has been found that the levels of radiocaesium in the underground parts of fungi (mycelium) are higher than the ^{137}Cs levels in the aboveground parts such as the medulla and the cap **(Vinichuk and Johanson, 2003)**. The distribution of radiocaesium in various above-ground parts of fungi is uneven, with higher activity found in fungi caps **(Heinrich, 1993; Mukhopadhyay et al., 2007)**. Mushroom samples collected in coniferous forests are characterised by a high content of radionuclides compared to mushroom samples collected in deciduous forests **(Čipáková, 2004)**. ^{137}Cs activity ranged between 273 and 1.165 Bq kg^{-1} in fresh mushrooms collected in the French Alps in 1999 – 2002 (**Pourcelot et al., 2003)**. In contrast, the highest contamination measured in the dry matter of mushrooms originating in the Bohemian-Moravian Highlands (Czech Republic) was 2.263 Bq kg^{-1}. Contamination levels in Slovakia were significantly lower **(Dvořák et al., 2006)**.

Research conducted in Europe, Japan and North America in 2008 has shown that *Boletus subtomentosus* is the species with the highest contamination levels of the entire Boletus family. The potential danger represented by this mushroom is even bigger due to its presence in large numbers in all types of forests **(Duff and Ramsey, 2008)**. However, we could not find this mushroom species in the Bobrůvka River Valley.

The mushrooms analysed in our study were collected from a small area in pine tree habitats in areas of steeply sloping hillsides modelled by river erosion. The results for ^{137}Cs mass activities are shown in **Table 1**. The largest ^{137}Cs activity was found in the species *Boletus edulis*, amounting to 1.575 Bq kg^{-1} in native state, which is just below the legal limit. However, this species also showed the greatest variation range, as mushrooms with a mass activity of 28 Bq kg^{-1} were also found. In contrast, the greatest activity seen in *Imleria badia*, which as a representative of the Boletaceae family is often quoted in connection with the highest cumulative ability, was only 316 Bq kg^{-1}. The differences between the average contamination values of both mushroom species (197 and 173 Bq kg^{-1}) were not, however, significant due to high variability. **Kunová, Dvořák and Beňová (2006)** reported the highest ^{137}Cs activity value measured in *Imleria badia* from the Staré Ransko locality (the Bohemian-Moravian Highlands). This activity reached 708 Bq kg^{-1}, which is around twice that of the mushrooms studied in our work. Another representative of the Boletaceae family is *Xerocomellus chrysenteron*. The variation range is from 19 to 2 Bq kg^{-1}, which shows a very low ability to accumulate radiocaesium. Given the 33 specimens monitored, this cannot be a coincidence. The results for *Neoboletus luridiformis* are also surprisingly low. In the 7 specimens found, the maximum concentration of contamination measured was 73 Bq kg^{-1}.

Table 1 Mass activity [Bq kg^{-1}] ^{137}Cs in different species of mushrooms.

Mushroom species	n (number)	Mean Bq kg^{-1}	CNS* Bq kg^{-1}	Max Bq kg^{-1}	Min Bq kg^{-1}
Imleria badia	113	**173**	9.7	316	11
Boletus edulis	19	**197**	12.9	575	28
Neoboletus luridiformis	7	**27**	2.7	73	10
Xerocomellus chrysenteron	33	**13**	1.5	19	2
Suillus grevillei	1	**28**	7.4		
Russula aeruginea	29	**27**	1.9	39	9
Russula olivacea	3	**18**	2.4	5	24
Russula vinosopurpurea	2	**13**	1.7	5	20
Russula ochroleuca	1	**9**	1.3		

Note: * Average combined standard uncertainties u_a (Guide to the Expression of Uncertainty in Measurement for Standardization).

Furthermore, the dependence between the average weight of the mushrooms and their activity was also studied. For *Imleria badia*, the highest activity level of 316 Bq kg^{-1} was measured in a mushroom sample weighing 25 g, while the lowest activity level of 56 Bq kg^{-1} was measured in a mushroom sample weighing 24 g. The mushroom sample with the lowest weight (8 g) had an activity level of 106 Bq kg^{-1}.

CONCLUSION

The results show that in the monitored area *Boletus edulis* shows a higher capacity of ^{137}Cs contamination as compared to the more frequently occurring *Imleria badia*. No correlation was found between the radiocaesium mass activity and the size of the collected mushrooms. No ^{137}Cs activity above the limit was found in mushrooms in the monitored area in the years 2017 – 2018.

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EFFECTS OF THE ADMINISTRATION OF BREWED ROBUSTA COFFEE LEAVES ON TOTAL ANTIOXIDANT STATUS IN RATS WITH HIGH-FAT, HIGH-FRUCTOSE DIET-INDUCED METABOLIC SYNDROME

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ABSTRACT
Robusta coffee (*Coffea canephora*) leaves contain phytochemical compounds and have antioxidant and anti-diabetic effects. This study investigated the effect of brewed Robusta coffee leaves on the total antioxidant status in metabolic syndrome rats. Metabolic syndrome in rats was induced by high-fat-fructose diet containing pork oil (20%), cholesterol (1.5%), cholic acid (0.5%), standard feed (80%), and fructose (1 mL per 200 g BW). The animals were categorized into normal control group (K1), metabolic syndrome control group without treatment (K2), mangiferin treatment group (X1), brewed Robusta coffee leaves 0.09 g per 200 BW group (X2), brewed Robusta coffee leaves 0.18 g per 200 BW group (X3), and brewed Robusta coffee leaves 0.36 g per 200 BW group (X4). Each dose of the coffee leaves was brewed with 3.6 mL of water at 70 °C for 10 min. The intervention was administered for 28 days. There was a significant increase in the total antioxidant status (p <0.000) in all the groups. In conclusion, the administration of brewed Robusta coffee leaves increased the total antioxidant status in metabolic syndrome rats.

Keywords: metabolic syndrome; Robusta coffee leaves; total antioxidant

INTRODUCTION
Metabolic syndrome is a cluster of metabolic disorders characterized by hyperglycemia, hypertension, obesity, low high-density lipoprotein (HDL), and hypertriglyceridemia **(Srikanthan et al., 2016)**. Obesity and insulin resistance are the known risk factors for metabolic syndrome. The prevalence of metabolic syndrome has been increasing every year. From 2009 to 2013, the prevalence of metabolic syndrome increased from 28.84% to 30.52% in adults >30 years of age in Korea **(Lee et al., 2018)**. In Indonesia, its prevalence by the province in 1995 − 2007 was about 21.66%, with the highest prevalence noticed in Jakarta **(Herningtyas and Ng, 2019)**.

The main mechanism of obesity and insulin resistance is oxidative stress **(Hurrle and Hsu, 2017; Marseglia et al., 2015)**, which damages both insulin secretion by the pancreatic β-cells and glucose transport in the muscle and adipose tissues **(Marseglia et al., 2015)**. Oxidative stress is caused by increases in lipid peroxide, malondialdehyde (MDA), carbonyl protein, and oxide xanthine activity because of an imbalance between free radicals and antioxidants **(Mancini et al., 2015; Tangvarasittichai, 2015)**. The body has natural defenses in the form of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), dan glutathione peroxidase (GPx). Besides, consumption of a diet high in antioxidants provides defence against oxidative stress **(de Souza Cardoso et al., 2018)**. Robusta coffee leaves are consumed by the people of West Sumatra, Indonesia, as a healthy beverage **(Rasyid, Sanjaya and Zulharmita, 2013)**. These leaves contain caffeine, chlorogenic acid, flavonoid, and mangiferin, which have antioxidant and anti-diabetic properties **(Chen, Ma and Kitts, 2018)**.

In this study, we evaluated the effect of administration of brewed Robusta coffee leaves processed using the Japanese style green tea process (JGTP) **(Chen, Ma and Kitts, 2018)** and mangiferin on the total antioxidant status in a rat model of high-fat-fructose diet (HFFD)-induced metabolic syndrome.

Scientific hypothesis
Brewed Robusta coffee leaves increase the total antioxidant status in rats with metabolic syndrome.

MATERIAL AND METHODOLOGY
This study is included in the research project Study of The Administration of Brewed Robusta Coffee Leaves for Metabolic Response In Vivo in Metabolic Syndrome supported by funding received from Faculty of Medicine, Universitas Diponegoro 2019.

The processing of Robusta coffee leaves using JGTP

Robusta coffee leaves were picked from 2nd, 3rd, and 4th leaves of each branch of Robusta coffee plants. Leaves were sorted and then blanched for 75 s. They were then dipped in water and placed in a seducer for ±15 min to separate the leaves from the midrib. Next, the leaves were crushed using a crushing-tearing-curling machine 3 times. Finally, the leaves were dried for 4 − 5 h in a rack drier machine at 80 °C. The dried leaves were stored in airtight containers. The process was carried out in a mini-processing green tea processing laboratory at The Tea Quality Processing & Testing Laboratory in The Tea and Quinine Research Center Gambung, Bandung, Indonesia.

Animal treatments

Six-week-old male Wistar rats (n = 36), each weighing 150 – 200 g, were acquired from the Centre for Food and Nutrition of Universitas Gadjah Mada, Yogyakarta-Indonesia. The animals were provided standard feed of Comfeed II at 20 g per rat per day and water ad libitum. Body weight gain was recorded weekly and the remaining food was weighed daily. The experiments were approved by The Ethical Committee of Medical Research of Faculty of Medicine, Universitas Diponegoro (No. 16/EC/H/FK-UNDIP/III/2019), Indonesia. Rats were randomly divided into six groups (n = 6 per group): healthy control (K1), metabolic syndrome without treatment (K2), mangiferin 20 $mg.kg^{-1}$ BW (X1), brewed Robusta coffee leaves 0.09 g per 200 g BW (X2), brewed Robusta coffee leaves 0.18 g per 200 g BW (X3), and brewed Robusta coffee leaves 0.36 g per 200 gBW (X4) groups. All animals, except the K1 group animals, were fed a HFFD for 14 days. This diet contained pork oil (20%), cholesterol (1.5%), cholic acid (0.5%), and standard feed (80%) and was administered orally, while fructose 1 mL per 200g BW was administered by sonde. Metabolic syndrome was defined when the rats had fasting blood glucose ≥110 $mg.dL^{-1}$, triglycerides >150 $mg.dL^{-1}$, and HDL <40 $mg.dL^{-1}$.

Brewed Robusta coffee leaves were administered daily through a gastric sonde. The doses were brewed in 3.6 mL of water at 70 °C for 10 min. Mangiferin was dissolved in 3.6 mL of water and administered through a gastric sonde. The intervention was performed for 28 days.

Blood sample analyses

Fasting blood glucose, triglyceride, and HDL measurements as criteria of metabolic syndrome were performed after 14 days of HFFD administration. Fasting blood glucose, triglyceride, and HDL were analysed by GOD-PAP, GPO, and CHOD-PAP methods respectively. Measurement of total antioxidant status was performed before intervention and at the end of intervention. Total antioxidant status was analysed by ABTS method. Blood sampling to analyze fasting blood glucose, triglyceride, HDL, and total antioxidant status through plexus retroorbital. Blood serum was analyzed in the Centre for Food and Nutrition of Universitas Gadjah Mada Yogyakarta-Indonesia.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistic 22 software. Data are presented as mean ±SD or median. Paired *t*-test and one-way analysis of variance were used for parametric results; differences between the groups were evaluated using the *post-hoc* test. Wilcoxon, Kruskal-Wallis, or Mann-Whitney test was used, as appropriate, for non-parametric results.

RESULTS AND DISCUSSION

Oxidative stress occurs when more oxidizing species are produced than the amount of antioxidants present in the body **(Morillas-Ruiz and Hernández-Sánchez, 2015)**. Insufficient antioxidant levels in the body could be overcome by dietary antioxidants **(Mirończuk-Chodakowska, Witkowska and Zujko, 2018; Yadav et al., 2016)**.

In this study, body weight of the animals significantly increased after treatment in all the groups (p <0.05). The X2 group (34.33 ±1.03 g) experienced the highest weight gain after the administration of brewed Robusta coffee leaves compared to other treatment groups (Table 1). The X1 group (22.00 (22.00 – 26.00) g) also experienced weight gain. The weight gain was the highest in the K2 group (43.50 ±1.64 g). The body weight between all groups significantly different before and after treatment (Table 2). Statistically, the body weight in the X4 group was not significantly different from that in the K1 group, as well as the body weight in the X3 group was not significantly different from that in the X4 group. This indicated that the increase in body weight in the X3 group was similar to that in the K1 group.

HFFD administration in rats has been known to cause weight gain and increases in blood glucose, triglyceride, LDL, and cholesterol levels **(Nugroho et al., 2012)**. In this study, rats were administered HFFD for 14 days to achieve metabolic syndrome. In a previous study, HFFD administration caused hyperglycemia and dyslipidemia **(Octavia, Djamiatun and Suci, 2017)**. In another study, a high-fat diet increased fasting blood glucose and a high-fructose diet increased triglyceride levels in rats **(Huang et al., 2004)**. The administration of a high-fat diet increased reactive oxygen species (ROS) and decreased antioxidant enzyme activity **(Du et al., 2012; Sreekumar et al., 2002)**. A high-fructose dietary induces oxidative stress by decreasing the antioxidant defense system **(Zhang, Jiao and Kong, 2017)**.

Rats achieved metabolic syndrome condition with fasting blood glucose (131.14 ±2.13 – 132.70 ±1.48 $mg.dL^{-1}$), triglycerides (153.49 ±1.96 – 157.18 ±4.88 $mg.dL^{-1}$), and HDL (24.59 ±1.99 – 26.22 ±1.69 $mg.dL^{-1}$) as seen in Table 3. Fasting blood glucose and triglyceride levels in the K2, X1, X2, X3, and X4 groups were increased compared to those in the K1 group. Meanwhile, HDL level in the K2, X1, X2, X3, and X4 groups was decreased compared to that in the K1 group.

Table 1 The Average Body Weight Before and After Treatment.

Groups	Body Weight (g)		Δ	*p*	*P*
	Pre	Post	Mean ±SD		
	Mean ±SD	Mean ±SD			
K1 (n=6)	183.00 ±2.82	208.50 ±3.27	25.50 ±1.37	0.000[a*]	0.000[c*]
K2 (n=6)	195.83 ±2.04	239.33 ±2.16	43.50 ±1.64	0.000[a*]	
X1 (n=6)	197.50 ±5.68	220.50 ±5.95	22.00 (22.00 – 26.00)[d]	0.024[b*]	
X2 (n=6)	196.50 ±3.78	230.83 ±3.37	34.33 ±1.03	0.000[a*]	
X3 (n=6)	198.33 ±4.67	226.83 ±4.99	28.50 ±1.87	0.000[a*]	
X4 (n=6)	201.67 ±3.32	228.17 ±4.70	26.50 ±1.87	0.000[a*]	

Note: normal control group (K1); metabolic syndrome control group without treatment (K2); mangiferin treatment group (X1); brewed Robusta coffee leaves 0.09 g/200BW group (X2); brewed Robusta coffee leaves 0.18 g/200BW group (X3); brewed Robusta coffee leaves 0.36 g/200BW group (X4); a* = paired t-test $p<0.05$ = significantly different; b* = Wilcoxon test $p<0.05$ = significantly different; c* = kruskal-wallis test $p<0.05$ = significantly different; d = abnormal distribution data, displayed in median (min-max).

Table 2 Mann-Whitney Test Results for Weight Change Before and After Treatment.

Groups	Δ BW (g) Mean ±SD	*p* Value					
		K1	K2	X1	X2	X3	X4
K1	25.50 ±1.37	-	0.004*	0.026*	0.004*	0.019*	0.219
K2	43.50 ±1.64		-	0.003*	0.004*	0.004*	0.004*
X1	22.00 (22.00 – 26.00)			-	0.003*	0.004*	0.011*
X2	34.33 ±1.03				-	0.004*	0.004*
X3	28.50 ±1.87					-	0.122
X4	26.50 ±1.87						-

Note: normal control group (K1); metabolic syndrome control group without treatment (K2); mangiferin treatment group (X1); brewed Robusta coffee leaves 0.09 g/200BW group (X2); brewed Robusta coffee leaves 0.18 g/200BW group (X3); brewed Robusta coffee leaves 0.36 g/200BW group (X4); *$p<0.05$ = significantly different.

Table 3 Fasting Blood Glucose, Triglyceride and HDL Level After Administration of HFFD.

Groups	Fasting blood glucose (mg/dl)	Triglyceride (mg/dL)	HDL (mg/dL)
	Mean ±SD	Mean ±SD	Mean ±SD
K1 (n = 6)	71.29 ±1.53	68.77 ±5.97	86.36 ±2.28
K2 (n = 6)	132.70 ±1.48	157.18 ±4.88	25.05 ±1.84
X1 (n = 6)	131.81 ±1.88	153.75 ±3.11	26.22 ±1.69
X2 (n = 6)	131.56 ±2.57	156.12 ±2.48	26.22 ±1.30
X3 (n = 6)	131.14 ±2.13	153.49 ±1.96	26.22 ±2.15
X4 (n = 6)	132.53 ±2.36	156.12 ±2.77	24.59 ±1.99

Note: normal control group (K1); metabolic syndrome control group without treatment (K2); mangiferin treatment group (X1); brewed Robusta coffee leaves 0.09 g/200 BW group (X2); brewed Robusta coffee leaves 0.18 g/200 BW group (X3); brewed Robusta coffee leaves 0.36 g/200 BW group (X4).

Compared to the baseline level, a significant difference in TAS levels was found after the administration of brewed Robusta coffee leaves and mangiferin in the K1 (p = 0.010), X1 (p = 0.000), X2 (p = 0.027), X3 (p = 0.027), and X4 (p = 0.000) groups. Meanwhile, there was no significant difference in TAS in the K2 group (p = 0.063) before and after treatment. Significantly increased TAS levels were found in the X2, X3, and X4 groups, implying that three doses of brewed Robusta coffee leaves could significantly increase TAS levels in rats with metabolic syndrome (Table 4).

After the administration of brewed Robusta coffee leaves, the TAS increased in the treatment groups. The higher the doses administered, the greater was the increase in the TAS in the metabolic syndrome rats. Rats administered mangiferin also experienced an increase in the TAS. This may be attributed to the presence of phytochemical contents, such as mangiferin, flavonoids, chlorogenic acid, and caffeine, in brewed Robusta coffee leaves. The processing of Robusta coffee leaves by the JGTP method contributes to the retention of more number of phytochemical components **(Chen, Ma and Kitts, 2018)**. In previous study, the extract of Robusta coffee leaves has high antioxidant activity. It is equivalent to the content of phenolic components in the leaves. The phenolic components contained in leaves contributes significantly to antioxidant capacity **(Kristiningrum, Cahyanti and Wulandari 2017)**.

Table 4 Total Antioxidant Status Before and After Treatment.

Group	TAS level (mmol/L)		Δ		
	Pre	Post	Mean ±SD	*P*	*p*
	Mean ±SD	Mean ±SD			
K1 (n=6)	2.25 ±0.17	2.06 ±0.90	−0,19 ±0,11	0.010[a*]	0.000[C*]
K2 (n=6)	0.29 (0.15 – 0.44)[d]	0.22 (0.15 – 0.29)[d]	−0.14 (−0.15 – 0.00)	0.063[b*]	
X1 (n=6)	0.29 ±0.12	1.54 ±0.20	1.24 ±0.20	0.000 [a*]	
X2 (n=6)	0.26 ±0.10	0.66 ±0.15	0.44 (0.29 – 0.45)	0.027[b*]	
X3 (n=6)	0.26 ±0.10	1.31 ±0.22	0.74 (0.73 – 1.03)	0.027[b*]	
X4 (n=6)	0.26 ±0.10	1.37 ±0.20	1.10 ±1.18	0.000[a*]	

Note: The rats were classified into the following groups: normal control group (K1); metabolic syndrome control group without treatment (K2); mangiferin treatment group (X1); brewed Robusta coffee leaves 0.09 g/200 BW group (X2); brewed Robusta coffee leaves 0.18 g/200 BW group (X3); brewed Robusta coffee leaves 0.36 g/200 BW group (X4).
a* = paired *t*-test $p < 0.05$ = significantly different; b* = Wilcoxon test $p < 0.05$ = significantly different; c* = Kruskal–Wallis test $p < 0.05$ = significantly different; d = abnormal distribution data, displayed in median (min-max).

The phytochemical caffeine primarily contributes in improving the antioxidant status. The antioxidant effect of caffeine is exerted by scavenging the hydroxyl radicals **(Yamagata, 2018)**. Caffeine directly inhibits lipid peroxidation and has a high inhibitor level against radical formation. This compound can also reduce oxidative stress and ROS, as well as protect antioxidant system **(Jeszka-Skowron et al., 2016; Tellone et al., 2015)**. The administration of caffeine at 30 and 100 $mg.kg^{-1}$ reduces lipid peroxidation and increases antioxidant enzyme activity **(Demirtaş et al., 2012)**. Consumption of caffeine 5 $mg.kg^{-1}$ body weight per day in 2 doses daily can reduce MDA and elevate the total antioxidant capacity **(Metro et al., 2017)**.

Chlorogenic acid also acts as an antioxidant. It donates hydrogen atoms to reduce free radicals and inhibits oxidation reactions. It also oxidizes phenoxyl radicals and stabilizes them through resonance stabilization **(Liang and Kitts, 2016)**. Chlorogenic acid at 5 $mg.kg^{-1}$ for 45 days causes a decrease in lipid oxidation and an increase in antioxidant endogenous enzymes in diabetic rats **(Pari, Karthikesan and Menon, 2010)**. High chlorogenic acid shows a high level of efficiency in scavenging DPPH radicals and converting Fe^{3+} to Fe^{2+}. This compound shows antioxidant activity by donating hydrogen to free radicals **(Liang and Kitts, 2014; Wu, 2007)**. 5-O-caffeoylquinic acid (5-CQA) is a subclass of chlorogenic acid that has a strong hydroxyl radical scavenger activity with a constant scavenger rate HO of $7.73 \times 109\ M^{-1}\ s^{-1}$ **(Liang and Kitts, 2014)**.

Flavonoids are part of antioxidants that contribute to the high antioxidant capacity and are present in fruits and vegetables. High flavonoid intake is associated with high plasma levels of total antioxidant capacity **(Alipour, Rashidkhani and Edalati, 2016)**. Intake of flavonoids, such as flavan-3-ols, flavonols, and anthocyanins, decreases dyslipidemia, induces antioxidant capacity, and prevents insulin resistance in diabetic patients **(Yamagata, 2019)**. Patients with type 2 diabetes administered flavonoid-enriched chocolate at 27 g per day for a year reduced insulin resistance and improved insulin sensitivity **(Curtis et al., 2012)**. The administration of the flavonoid quercetin at 15 mg per kg per day for 4 weeks decreased lipid peroxidation and increased antioxidant enzyme activity in diabetic rats **(Coskun et al., 2005)**. Mangiferin is a xanthone that is found in high levels in several parts of the mango. It is also found in Arabica coffee leaves and is thought to be found in Robusta coffee leaves **(Chen, 2019)**. Mangiferin confers its antioxidant effects by having a high iron-chelating ability **(Imran et al., 2017)**. The administration of mangiferin at 50 and 100 $mg.kg^{-1}$ reduces MDA levels in plasma and cardiac tissues and increases the level of SOD in cardiac tissues **(Arozal et al., 2015)**. In previous study, mangiferin at the dose of 10 $mg.dL^{-1}$ and 20 $mg.dL^{-1}$ in diabetic rats increased antioxidant defense mechanism, such as SOD and catalase **(Muruganandan et al., 2002)** Mangiferin at 40 mg per kg per day significantly reduced blood glucose levels and increased plasma insulin levels and antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase. Mangiferin from *Salacia chinensis* prevents oxidative stress and protects pancreatic β-cells in rats with streptozotocin-induced diabetes **(Sellamuthu et al., 2013)**.

CONCLUSION

The administration of brewed Robusta coffee leaves processed by the JGTP method increases TAS in rats with HFFD metabolic syndrome. The intervention of brewed Robusta coffee leaves with a dose of 0.36 g per 200 BW is the most effective dose in increasing TAS levels.

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PERSONALIZED NUTRITION AND "DIGITAL TWINS" OF FOOD

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ABSTRACT

Mathematization of research is one of the most effective methods of virtual substantiation of foodstuff recipe and technology. This approach allows creating a product that meets consumer's individual needs, i.e. personalized foodstuff (ethnicity, cultural preferences, regional and environmental characteristics, lifestyle), and at the same time reducing the time and cost of decision-making. The article discusses the hypothesis that the "digital twin" of a food product is a virtual model of the product, namely its mathematical model (simulation model). A simulation model is a logical and mathematical description of a food product that is used to conduct a computerized experiment in order to design desired characteristics and properties. The "digital twin" combines all variety of factors from chemical composition, functional and technological properties to organoleptic indicators. The application of the "digital twin" model of the foodstuff will allow: (1) reacting quickly to changes in the composition, properties and types of raw ingredients, (2) adjusting the product recipe in response to changes in consumer preferences, (3) designing products with a given chemical composition, nutritional value and functional orientation, (4) creating functional, specialized products taking into account the metabolism of nutrients (ethnicity, cultural preferences, health status and clinical factors). Products adapted to the needs of small categories of people will help reducing the risks for those who already have diseases, and will meet the needs of those who would like to make their diet more appropriate to individual needs. The proposed approach to creating a model of the "digital twin" of the foodstuff includes several stages. The first stage involves optimization of the nutritional and biological value of the designed product. The second stage is related to designing the food product's structural forms. But even if the recipe of a food product is optimally selected in the first stage, it does not guarantee its transformation during processing into a stable system with the required structural, mechanical, functional and technological parameters. Evaluation of the developed food product's efficiency is possible only by analysing numerous and various parameters and indicators. It is convenient to generalize (convolute) many parameters and indicators into a single quantitative dimensionless indicator. To assess the quality and adequacy of the food product, it is suggested to use an integral indicator in the form of additive convolution – the 'functional' of the food product quality.

Keywords: simulation model; personalized nutrition; digital twin; food product; integral indicator

INTRODUCTION

Personalized nutrition pursues the idea of individualization **(Ordovas et al., 2018)**, thus, recommendations and nutritional advice should not be based on the average norms of nutritional intake applied to gender and age groups of population differentiated by the level of physical activity.

Personalized nutrition, as other scientific fields at an early stage, applies many concepts and descriptors, for example, accurate nutrition, multi-layer nutrition, and individual nutrition.

As we move from the stratified to personalized and high-accuracy nutrition, it becomes necessary to apply more and more indicators or characteristics to achieve the desired goal. For example, stratification can be performed using one or more parameters, such as age, gender, or health status. Personalized nutrition needs to take into account the complexity of relations between the individual diet and phenotype, thus, a wide range of indicators will need to be used to achieve the goal of accurate nutrition, possibly including "big data" approaches.

At this stage of research, the authors adhere to the concept of creating products for the "individually selected diet".

In 2016, Japan introduced the concept of 'Society 5.0', which offers more comprehensive and more advanced application of digital technology in all areas of human life. According to this strategy, advanced technologies, after having been introduced into all spheres of life, should lead to the emergence of new forms and types of business.

One of the Sustainable Development Goals for Society 5.0. is to improve nutrition through the use of 'smart products' produced by advanced biotechnological methods.

To develop "smart" foodstuff that have a functional effect on the human body, levelling the risks of disease, it is necessary to take into account many different factors (physical and chemical, structural and mechanical, functional and technological, etc.). "Playing" with (imitating) all possible structural relations and restrictions is possible only with the use of a virtual product model.

Grieves (2014) in his work notes that the term 'digital twin' appeared in 2003 as part of reading for the discipline 'Product Lifecycle Management' at the Florida Institute of Technology (https://www.fit.edu/).

The term digital twin has numerous definitions **(Bolton et al., 2018; Claessgen and Stargel, 2012; El Saddik, 2018; Lee, Bagheri and Kao, 2015; Söderberg et al., 2017; Tao et al., 2018)**.

For a digital twin food product, the definition of **Söderberg et al. (2017)** is relevant, objective and realistic – 'using a digital copy of a physical system for real-time optimization'.

Solving the problems of an individual (personalized nutrition) taking into account many parameters, such as health status, lifestyle, clinical factors, ethnicity, cultural preferences, etc., may be associated with the development of specialized foods.

The authors of the article propose an approach to building a digital twin food product, using the example of an *anti-sclerotic meat product*.

Scientific hypothesis

This article discusses the hypothesis that the "digital twin" of a food product is a virtual model of this product, namely its mathematical model (simulation model), which combines the whole variety of factors from the chemical composition, functional and technological properties to organoleptic indicators. A simulation model is a logical and mathematical description of an object that can be used for digital experiments on a computer in order to design, analyse and evaluate the functioning of this object **(Mukha, 2010)**.

Using the digital twin of a food product before its launch into mass production, technological engineers can analyse the nutritional, biological and energy value and other characteristics of the product.

The virtual simulation model will allow technologists reacting to changes in the physical and chemical composition of raw materials used or replacement of main or auxiliary raw materials in a real time mode, and accordingly adjusting the recipe to obtain a product with the specified chemical composition and guaranteed quality.

The "digital twin" will allow scientists obtaining interpolation and extrapolation data without conducting additional laboratory studies with a 95% confidence level (or $p < 0.05$)

MATERIAL AND METHODOLOGY

As an example of applying the virtual modelling approach, the task was set to create a digital twin of a meat product for the functional nutrition from the hearts and aortas of pigs, intended for dietary nutrition, in order to reduce the risk of developing and preventing hyperlipidemia and atherosclerosis. Step-by-step scientific experiment and laboratory research have been described **(Kotenkova and Chernukha, 2019; Chernukha, Fedulova and Kotenkova, 2018)**.

Another object was a meat food product enriched with iodine for school-age children.

When designing these products, we are interested in the best solution to the problem, taking into account the metabolism of nutrients (ethnicity, cultural preferences, health status, lifestyle, and clinical factors). The indicator that characterizes the quality of the task is called the objective function or criterion of optimality. The general optimization problem is formulated as follows: to select for variables $x_1, x_2, \ldots, x_n$ the appropriate values that provide the extremum of the objective function **(Mastyaeva, Goremykina and Semenikhina, 2016)**.

If the objective function and the constraint system are non-linear, the optimization problem is called a non-linear programming problem.

To solve non-linear programming problems, various iterative procedures are used to find the optimum: the gradient method, the coordinate descent method and the steepest descent method.

The software implementation of the models is possible in the universal simulation systems Simplex3, AnyLogic, etc. **(Schmidt, 2001; Ivashkin, 2016; Karpov, 2005)**.

RESULTS AND DISCUSSION

We will consider the mechanism for creating a "digital twin" of a food product, using the example of modelling the recipe and technology of a meat product for elderly nutrition (option 1) and baby food (option 2).

One of the main purposes while creating a functional and/or specialized diet is to provide the nutrients missing in the diet, as well as to reduce the risk of developing and preventing a particular disease.

Formulation of the problem's structural optimization, at different levels of the technology description of the digital twin food product, is reduced to minimizing the deviation of the actual parameters from the specified normative (reference, desired) values by finding a balance for the selected indicators between the input and output flows of the material.

The digital twin of the food product will allow employees of food industry enterprises to respond quickly to changes in the properties and types of raw ingredients, to respond to changes in consumer preferences and to create products with a predetermined chemical composition, nutritional value and functional orientation to a particular category of consumers. The optimal solutions to these problems in the design of food products can be achieved using formalized mathematical descriptions – mathematical models that reflect in an analytical way the set of functional relations between the technological, economic and other parameters inherent to the raw ingredients, the required characteristics of the finished product (objective function or optimization

criterion) and a number of restrictions arising from regulatory documentation requirements.

The first stage is related to the analysis of raw materials.

Option 1 is used to level out pathological processes and reduce the risk of hyperlipidaemia and atherosclerosis.

Based on the initial conditions for *the raw meat* – protein content at least 18%; fat content not more than 15%; the presence of tissue-specific peptides with molecular weights 809.4 ±1.0, 776.5 ±1.0, 765.6 ±1.0, 739.2 ±1.0, 710.8 ±1.0, 229.2 ±1.0, 162.1 ±1.0, 156.0 ±1.0, 148.1 ±1.0, 140.2 ±1.0 and 133.1 ±1.0 kDa; the presence of Apo 1 (which participates in the formation of high-density lipoproteins) or the presence of pre-Apo A-1 (which participates in the suppression of oxidative stress) – pork aorta and pork heart were chosen as the main raw material samples.

Option 2 is used to eliminate deficient conditions – hypovitaminosis, lack of trace elements (in particular iodine), leading to the development of a number of diseases. The amino acid methionine (binds 56 – 60% of iodine) must be present for the absorption of iodine from the fatty acid-iodine complex in the human body.

Restrictive parameters for the development of the product formulation for the raw meat are protein content of not less than 16% and fat content of not more than 16%; for vegetable oils – vitamin E of not less than 10 mg; for vegetable raw materials – content of β-carotene of not less than 5 mg; for iodine-containing raw materials – iodine content of not less than 0.1%.

As a result of the operator's work with the database, a list of raw materials of animal and plant origin that can be used in the formulation of an iodine-enriched product under the above boundary conditions was obtained.

For baby food, it is possible to use raw meat such as the first-category beef and pig meat (the choice is related to the main criterion – the presence of amino acids: tyrosine, phenylalanine, proline and histidine; PUFA).

For vegetable oils - soy oil, corn oil, which contains a sufficient amount of linoleic and linolenic acids along with a high content of vitamin E, as well as sunflower oil and peanut oil due to the high content of vitamin E and PUFA.

When choosing a plant component – a source of β-carotene, the choice of red carrots is obvious because of the high content of β-carotene in its composition. Along with this, the amino acid and vitamin composition of carrots contains histidine, phenylalanine, tyrosine and proline, which are necessary for a young body and contribute to better absorption of iodine.

As an iodine-containing raw material, fucus extract is used – a product of brown fucus seaweed processing, obtained by water extraction of brown fucus seaweed (*Fucus vesiculosus* L.), followed by the distillation of the extractant and obtaining a dry extract by spray drying.

Varying the structural ratio of the recipe components within the present restrictions, we see a way of changing the nutritional and biological values of the product, the ratio between protein and fat, etc.

When designing functional products, for example a meat gerodietic product, one of the main restrictions in optimization problems arises from the amino acid conformity criterion of protein that determines the ratio of mass fractions of amino acids such as methionine, cystine, tryptophan and lysine, taking into account the roles of isoleucine, leucine, phenylalanine and tyrosine as gerontological competitors of tryptophan **(Ivashkin and Nikitina, 2016)**.

The mathematical record of the criterion is as follows:

$$K = \frac{a_{(Met+Cys)}}{a_{Lys} \cdot C_{Trp}} \cdot \frac{\sum_{j=1}^{4} a_{jn}}{\sum_{j=1}^{4} a_{jэ}} = 1$$

Where: K is the coefficient of amino acid conformity, fractional units; $a_{(Met+Cys)}$ and a_{Lys} are the mass fraction of the amino acids methionine + cystine and lysine, g/100 g protein; C_{Trp} is the score of the amino acid tryptophan in the protein of the gerodietic product in relation to the FAO/WHO standard, fractional units; $a_{jn}, a_{jэ}$ are the mass fractions of the j^{th} amino acids in the protein and the FAO/WHO standard, respectively, g/100 g of protein; and index j identifies, respectively, the amino acids: 1 – isoleucine, 2 – leucine, 3 – phenylalanine and 4 – tyrosine.

Using this criterion (ideally K = 1), it is possible to quantify the protein composition of the designed gerodietetic products.

The biomedical requirement for gerodietic products in terms of the mass fraction of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids is that they should correspond to a ratio of 3:6:1. In a mathematical arrangement, the task looks as follows:

$$\sum_{k=1}^{7}\sum_{j=1}^{m} q_{kj} l_j x_j + \sum_{k=8}^{10}\sum_{j=1}^{m} q_{kj} l_j x_j < 9 \sum_{k=11}^{13}\sum_{j=1}^{m} q_{kj} l_j x_j$$

Where:

q_{kj} is the mass fraction of the k^{th} fatty acid in the fat of the j^{th} recipe component, %; l_j is the mass fraction of fat in the j^{th} component of the recipe, %; x_j is the mass fraction of the j^{th} fat-containing component of the recipe, %; and the coefficient k is 1 – 7 for SFA, 8 – 10 for MUFA and 11 – 13 for PUFA accordingly.

Meat raw materials are multicomponent and variable in composition and properties. This can lead to significant fluctuations in the quality of the finished product. In this regard, it is very important to know the functional and technological properties (FTP) of various types of basic raw materials and their components, to understand the role of auxiliary materials and the essence of FTP changes under the influence of external factors.

Thus *the second stage* is related to designing the structural forms of a food product. The optimal formulation of a food product recipe at the first stage does not guarantee its conversion into a stable system with the required structural-mechanical and functional-technological parameters during technological processing.

Foods have individual structural forms (consistency, appearance, binding coherence, texture, etc.) due to peculiarities in the occurrence of protein–protein, protein–water, protein–fat and water protein–fat types of colloidal chemical processes.

Table 1 Major functional and technological properties of some raw meats **(Zharinov, 1994)**.

Type of raw meat	Before thermic processing							
	Moisture-binding capacity, % of the total moisture		Plasticity, $\times 10^{-1}$ m^2/kg		Water absorption, % of the initial mass		Fat absorption, % of the initial mass	
	3 mm	Mince cutter	3 mm	Mince cutter	3 mm	Mince cutter	3 mm	Mince cutter
Beef, 2nd grade	78.0 ±2.8	87.0 ±5.0	9.74 ±0.4	11.76 ±0.8	56.2 ±4.4	55.3 ±3.8	28.4 ±3.0	30.9 ±1.6
Semi-fat pork	73.8 ±6.0	74.6 ±3.8	9.46 ±0.3	11.5 ±0.5	34.9 ±2.3	64.2 ±4.2	22.5 ±1.2	28.6 ±2.1
Fat pork	-	-	10.52 ±0.2	12.31 ±0.3	29.2 ±2.4	32.2 ±2.6	19.8 ±2.0	23.4 ±1.7
Beef honeycomb	49.8 ±3.7	65.4 ±4.2	5.8 ±0.6	7.7 ±0.4	53.2 ±4.4	58.3 ±3.8	26.1 ±2.2	37.8 ±2.5
Beef lungs	94.3 ±1.6	96.0 ±1.2	11.3 ±0.9	11.5 ±0.8	30.6 ±2.8	35.0 ±4.0	25.2 ±2.2	32.8 ±3.1
Beef spleen	66.6 ±3.4	64.2 ±4.0	18.1 ±0.4	18.8 ±0.3	26.0 ±2.0	29.3 ±2.7	16.6 ±1.1	18.2 ±0.8
Beef oesophagus meat	78.2 ±3.3	80.2 ±2.8	8.1 ±0.6	8.7 ±0.4	15.1 ±1.1	16.5 ±1.4	9.8 ±0.9	12.2 ±1.3
Cattle snout	100 ±0.0	100 ±0.0	4.2 ±0.5	4.8 ±0.5	5.1 ±0.6	6.1 ±0.6	9.34 ±0.8	9.8 ±0.4

The protein component of the recipe usually acts as the main structure-forming agent in meat systems (even with its high quantitative content and balanced amino acid composition). Protein can manifest its native properties to various degrees depending on its origin (muscle, connective tissue, vegetable, milk, etc.), structure (fibrillar, globular), environmental conditions (pH, temperature, ionic strength) and many other indicators (degree of raw material mincing, depth of autolytic changes, etc.) **(Zharinov, 1994)**.

To implement the second stage, it is necessary to have information about the actual FTP values of the main raw materials **(Červenka, Frühbauerová and Velichová, 2019)**, auxiliary ingredients, the kinetics of biochemical and colloid-chemical processes (structuring – first of all) in multicomponent food systems, and the analytical and

Along with the introduction of increasingly convenient tools for big data processing and storage, it becomes possible to increase the frequency of use and number of alternatives for creating a digital twin food product, which in its turn increases the reasonableness and feasibility of decisions.

The following mathematical formulas are used to calculate the FTP of multicomponent products:

1) model of the water-binding capacity (WBC):

$$WBC = \sum_{i=1}^{N} w_i x_i$$

where w_i is the WBC of the i^{th} prescription component;

2) model of the fat-binding capacity (FBC):

$$FBC = \sum_{i=1}^{N} l_i x_i$$

where l_i is the FBC of the i^{th} recipe component;

3) model of the water-holding capacity (WHC):

$$WHC = \sum_{i=1}^{N} v_i x_i$$

where v_i is the WHC of the i^{th} recipe component;

4) model of the critical shear stress (CSS):

$$CSS = \sum_{i=1}^{N} q_i x_i$$

empirical dependencies that characterize the basic laws of heterogeneous dispersed systems with varying physico-chemical factors.

Food product databases now should contain information not only on the main parameters – moisture, protein, fat content, energy value, and amino acid, fatty acid, vitamin and mineral composition, but also on the FTP of raw materials of animal and vegetable origin.

As an example, we give the main characteristics of the FTP of some kinds of protein-containing raw materials (Table 1). They can be used to determine the compatibility conditions of the components in a formulation and optimize the choice of ingredient ratios, taking into account the probability of mutual regulation of the properties both of individual components and of the whole system obtained as a result.

where q_i is the value of the CSS of the i^{th} recipe component;

5) model of the dynamic viscosity (η):

$$\eta = \sum_{i=1}^{N} \frac{V_i x_i}{\eta_i}$$

where η_i is the η of the i^{th} recipe component; and V_i is the volume fraction of the recipe component;

6) model of density (ρ):

$$\rho = \left(\sum_{i=1}^{N} \frac{x_i}{\rho_i} \right)^{-1}$$

where ρ_i is the ρ of the i^{th} recipe component;

7) model of the active acidity indicator (pH):

$$pH = -\lg\left(\sum_{i=1}^{N} x_i 10^{-pH_i} \right)$$

where pH_i is the pH of the i^{th} recipe component; and x_i is the mass fraction of the i^{th} recipe component in the formulations presented here under numbers (1) to (7).

The employees of the Russian Research Institute of the Meat Processing Industry (VNIIMP) (http://www.vniimp.ru) have developed a method to define WHC, fat holding capacity (FHC) and minced meat stability (MMS):

$$WHC = \left(Wm_1 M_1 - Mm_2 \right) \cdot 100;$$

$$FHC = \left(Fm_1 M_1 - Mm_3 \right) \cdot 100;$$

$$MMS = \left[\frac{(m-M)}{m_1}\right]\cdot 100;$$

where W is the moisture content in minced meat, %; F is the fat content in minced meat, %; M is the mass of all separated broth with fat, g; M_1 is the mass of the tested broth with fat, g; m_1 is the weight of minced meat suspension, g; m_2 is the mass of water in the tested broth, g; and m_3 is the mass of fat in the tested broth, g.

The most crucial step in obtaining guaranteed quality in the production of meat products is the heat treatment stage.

To calculate thermograms of heat treatment of meat products, a mathematical model is used that describes a non-stationary field of temperatures containing the product. The mathematical model is a parabolic Fourier's thermal conductivity equation, in cylindrical coordinates with variable coefficients, with specified initial conditions (the initial temperature field in the product before the next stage of processing):

$$\frac{dU(r,t)}{dt} = a\frac{d^2U(r,t)}{dr^2} + \frac{1}{r}\cdot\frac{dU(r,t)}{dr} \quad 0 < r < R, \quad 0 < t < t_k$$

Initial conditions (product temperature before the heat treatment):

$$U(r,t) = U_0 = const, \quad t = 0 \quad 0 < r < R$$

Boundary conditions (on the side surface of the product):

$$\frac{dU(r,t)}{dr} = SU_{cp}(t) - U(R,t), \quad S = \frac{\alpha}{\lambda}, \quad r = R, \quad 0 < t < t_k$$

Conditions for the symmetry of geometry and heating in the center of the loaf

$$\frac{dU(0,t)}{dr} = 0, \quad U(0,t), \quad r = 0, \quad 0 < t < t_k$$

Where: U – current product temperature; U_0 – initial product temperature; U_{cp} – temperature of heating medium; $U_к$ – final product temperature; R – outer product radius; r – radial coordinate; α – heat transfer coefficient; λ – coefficient of thermal conductivity.

Bakery dough is also a complex system where yeast cells produce carbon dioxide during the loosening process. **Stanke**, **Zettel and Hitzmann, 2014** proposed a model that describes the loosening process by introducing the specific rate of CO_2 production as a variable and biomass as a parameter. Using different amounts of yeast at different temperatures, they modeled the process with an average percentage error of less than 0.5%

$$F(q_{co_2}, \eta, N_b) = \sum_{i=1}^{n}\left(V_{rel,i} - \hat{V}_{rel,i}\right)^2 + \left(P_n - \hat{P}_n\right)^2 = min$$

where q_{co_2} - CO_2 specific capacity; η - viscosity; N_b – amount of bubbles per volume; $V_{rel,i}, \hat{V}_{rel,i}$ - relative volume of bakery dough; $P_n, \hat{P}_n$ - porosity.

When producing dairy products, functional and structural characteristics must also be taken into account. The research (**Musina et al., 2018**) substantiates the reason for the swelling of whey protein extract in combination with various berry purees in the recipe of whipped frozen milk dessert at different temperatures.

The third stage is organoleptic assessment of the designed product by expert assessment methods with consistency testing.

Thus, the optimality of various structural options is analysed and verified on the basis of a comprehensive simulation model of a food product, i.e. on the digital twin.

Evaluation of the developed food product's efficiency is possible only by analysing the numerous heterogeneous indicators.

It is convenient to generalize (reduce) a set of indicators into a single quantitative dimensionless indicator. To do this, you have to enter a dimensionless scale for each of them, which must be the same for all the combined indicators. This technique makes them comparable.

One of the most common generalized indicators is the generalized Harrington function **(Harrington, 1965)**.

To assess the quality and adequacy of a food product, **Nikitina, Chernukha and Nurmukhanbetova (2019)** used the 'functional' – an integral indicator in the form of an additive convolution.

First of all, the functional determines the compliance with the set requirements for the chemical composition, FTP and organoleptic parameters. The functional reflects the weighted average total deviation of the actual values of the state parameters from the normative values. Taking into consideration the weighting indicators and the allocation of certain groups of indicators, this functional has the following form:

$$F(x) = 1 - \sqrt{\frac{1}{n}\sum_{i=1}^{n} a_i \sum_{j=1}^{n_i} b_{ij}\left(\frac{x_{ij} - x_{ij}^0}{\Delta x_{ij}^k}\right)} \to \max$$

Where n is the number of joined indicators; x_{ij}, x_{ij}^0 are the actual and desired values; Δx_{ij}^k is the limit deviation from the desired value for the k^{th} level of quality; b_{ij} is the weight coefficient of the j^{th} parameter in the i^{th} group; and a_i is an indicator of the group significance.

The value of the quality indicator changes from 1 (when the obtained values completely coincide with the recommended ones, i.e. best quality) to 0 when the lowest level of quality is reached (limit value), so that in the case of negative values of the functional, the product does not correspond to the specified quality level.

If all the considered indicators n are equal, then weight coefficients are determined by the formula:

$$b_{ij} = \frac{1}{n}$$

When determining weight coefficients at the inequality of weight coefficients one may use the method of expert estimates or the method of full factorial experiment, when columns of the response function (output parameter) y_{kr} of the r-th parallel repetition in the k-th experiment contain values: 1-0.7 – when the product features a very good quality level; 0.7-0.3 – good quality level; 0.3-0 – satisfactory quality level; 0-(-0.2) – poor quality level; less (-0.2) – very poor quality level.

CONCLUSION

When developing products for personalized nutrition, taking into account the metabolism of nutrients, it is necessary to take into account the whole variety of different variables and factors. This is almost impossible in a laboratory scientific experiment, and/or involves a lot of labor. It is possible to choose the optimal version of the recipe when "playing out" various situations in virtual space using a digital (computer, mathematical) model of the foodstuff.

The generalized mathematical model should include not only physical and chemical parameters (fat, protein, etc.), but also knowledge of dependencies of functional, technological, structural and mechanical characteristics that determine the conversion of the optimal formulation in the process of technological processing into a stable system.

Thanks to the use of mathematical modeling methods and a simulation model of the designed product, it is possible to obtain a "digital twin" of the foodstuff. 'New' technologies with the help of a digital twin make it possible to take into account the whole range of qualitative and quantitative indicators of meat products to develop recipes for new food products with a complex composition and characteristics, which allow non-drug prevention of diseases. Testing in the virtual world saves time, money and resources for physical scientific experiments.

Using an example of selected meat products, we showed the possibility of creating a digital model of the personalized foodstuff meeting all requirements.

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EFFECTS OF TRADITIONAL FISH PROCESSING METHODS ON THE PROXIMATE COMPOSITION AND PH OF FISH BLACK POMFRET (*PARASTROMATEUS NIGER*)

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ABSTRACT
Fish is an important food in many Iranian diets. This is a good source of protein. Fish is the main source of animal protein in Iran. The effects of three different traditional processing methods (freezing, brining and frying) on nutritive composition of halva sia fish stored under ambient room conditions were determined. Fresh halva sia fish were obtained from Behbahan fish market. Cooking and processing techniques were carried out on fish Halva sia Parastromateus niger. The proximate composition of raw Parastromateus niger was affected by cooking and processing techniques that were carried out by AOAC methods. Moisture contents decreased in fried and brined fillet while protein, fat and ash contents were significantly increased in fried fillet. The loss of moisture in fried and brined samples amounted to the highest levels; also the protein and fat value was proportionally high. The fish Parastromateus niger showed a decrease in their contents of moisture and fat as affected by frozen storage periods while ash and protein contents were increased after frozen storage periods. The nutritional value fish Parastromateus niger preserved until the end of the storage period.

Keywords: frying; brining; fish; *Parastromateus niger*; freezing; proximate composition

INTRODUCTION
Fish can be a source of protein for humans. In addition, with proper processing methods, it can also generate foreign exchange **(Mazrouh, 2015)**. The nutrients composition of fish is essential in fish processing, as it affects both its quality and technological properties **(Farid et al., 2014)**. In addition, conservation and processing can result in fish availability throughout the year **(Ben Smida et al., 2014; Oparaku, and Nwaka, 2013)**. Different methods of processing fish have different effects on their properties physico-chemicals, organisms and nutrients. **(Akinneye, Amoo, and Bakare, 2010)**. These effects can be caused by physicochemical changes, which affect digestion due to protein denaturation and reduced content of unsaturated fatty acids. The quality and shelf life of the fish varies with different methods and with acceptance by consumers **(Mojisola, 2014)**. Freezing and thawing have many effects on the physicochemical properties of frozen fish fillets. **Boonsumrej et al., (2007)** found that rapid shrimp freezing in cryogenic freezers at temperatures of -120 °C, which has very good effects on nutrient composition. Several changes have occurred during fish processing. Depending on the amount of heat and temperature, protein denaturation may result in exposure to reactive groups. Most proteins are compounds that can change in the heat process in terms of quality and quantity. The decrease solubility of temperature-sensitive protein can be used as an indicator of the time and temperature used in the heating process of fish species and fish products **(Akinneye, Amoo, and Bakare, 2010.; Mojisola, 2014; Boonsumrej et al., 2007; Alipour et al., 2010; Sarker et al., 2012)**.

El and Kavas (1996) has been reported that protein digestion to be reduced as a result of chemical reactions of the protein or protein with fat when boiling at high temperatures. **Pourshamsian et al., (2012)** reported that frying has the effects on the nutritional composition and properties of fish fatty acids and fish products. However, no basic information has been reported on the effects of processing methods on the nutritional and physical properties of fish processed in Eritrean fish processing plants. In general, different fish processing methods can include frostbite, freezing, salting, frying and boiling, and various combinations of this kind to give the fish a fresh and attractive form for the consumer and to provide long-term shelf life. These processing methods have many different applications and techniques and have a significant impact on the chemical, physical and nutritional composition of processed fish. This is because heating, freezing, and exposure to high salt concentrations lead to chemical and physical changes.

Figure 1 Fish Black pomfret (*Parastromateus niger*).

Finally, these methods can be of varying quality, so the subsequent effect on the shelf life of processed fish also varies **(Magnussen, Hemmingsen, and Vidar Hardarsson 2008; Lourdes et al., 2007)**. The cooking processes are widely carried out to increase the quality of seafood and to extend the shelf life of the products **(Talab, 2014)**. During the cooking process, some physical and chemical changes occur that decrease the nutritional value of the food. Thus, digestibility is increased due to protein denaturation in foods, while the content of thermo sensitive labile compounds, vitamins or unsaturated fatty acids is often reduced **(Alizade et al., 2009)**. The aim of current study was to follow up the changes which occur in proximate composition of Parastromateus niger by frying, brining and freezing techniques.

The preservation and processing can assure availability of fish in all year round **(Smida et al., 2014; Oparaku et al., 2013)**. The bio-chemical composition of fish is the vital aspect in fish processing, because which influences both the quality and technological properties of it **(Farid et al., 2014)**. Different processing methods of fish have different effect on their physicichemical and nutritional compositions **(Akinneye, et al., 2010)**.

Scientific hypothesis

The processing methods (freezing, brining and frying) have effects on nutritive composition and energetic values of fish *Parastromateus niger*.

MATERIAL AND METHODOLOGY

Sample collection and preparation

The fish samples with three replicates of Black pomfret (*Parastromateus niger*) (Figure 1) with an average weight of 1100-1700 g were procured from the Behbahan city local market, Iran. The fish were gutted and washed with the tap water. The sampled fish was cut down into sex parts; one as fresh sample was used and the three parts were used for freezing 7 , 21 and 35 days and a part was used for frying in oil and last part was used for brining process. All the samples were used for proximate analysis in laboratory.

Proximate analysis

The moisture, protein, lipid and ash content of the fish samples were determined by the **AOAC (2005)**.

Protein determination

Protein was determined using Kjeidahi method which involves digestion, distillation and titration as recommended by **AOAC (2005)**. The percentage protein was obtained using the formula: % Protein = N = 6.26 Where N= Nitrogen free extract.

Moisture determination

Moisture was determined using method as recommended by AOAC (2005). Percentage of moisture was calculated using the formula; % water = Wo – Wi/Wo x 100 Where; Wo = initial weight Wi = final weight.

Fat analysis

Fat was determined using soxhlet method as recommended by Association of official analytical **(AOAC, 2005)** the percentage fat will be obtained

The formula:

$$\% \text{ fat} = Wo - Wi/Wo \qquad (1)$$

Where:
Wo = Initial weight of fat,
Wi final weight of fat.

Determination of pH

A 5 g sample of the fish sample was homogenized in 40 ml of distilled water and the mixture was filtered. The pH of the filtrate was measured using a pH meter (Mettler Toledo 320-s, Shanghi, China) (Vynke, 1981).

Statistical Analysis

Chemical composition data were analyzed statistically using SPSS 15.0. The least significant difference test (LSD) at ($p \leq 0.05$) and Standard Error (Mean ± SE) were calculated.

RESULTS AND DISCUSSION

The effect of frozen storage on moisture, protein, fat, and ash contents of the fish were studied. The obtained results are tabulated in Table 1.

Table 1 showed the effects of storage at -18°C at different periods on the moisture contents of raw the fish. The initial moisture contents of raw, frozen 7 days, 21 days and 35 days fish samples were 84.72, 84.74, 74.65 and 74.18 %, respectively. The differences were duo to a gradual decreasing in their contents of moisture as affected by frozen storage different periods. With increasing storage time, moisture content decreased significantly. The loss of moisture in frozen fish samples can be attributed to the sublimation of ice in frozen storage and the loss of drop during the thawing process **(Abo Taleb, Sherif, and Ibrahim, 2011)**. Similar findings were reported for other species of fish such as Sea bass fillets **(Benjakul et al., 2005)**, Carp fish cutlets **(Surabhi and Das, 2007)**, Tilapia fillets **(Ibrahim and El-Sherif, 2008)**, *Labeo rohita* (**Gandotra et al., 2012)** and Catla fish cutlet **(Vanitha et al., 2013)**. The initial protein contents of raw, frozen 7 days, 21 days and 35 days fish samples were 7.67, 8.52, 18.64 and 20.89 %, respectively (Table 1). These differences were duo to increase in their protein content, which is affected by the frozen storage period. As storage time increased, protein content increased significantly.

The decreasing in fat content of raw, frozen 7 days, 21 days and 35 days fish samples might be due to oxidation and hydrolysis of fats which result in the formation of some volatile chemicals as aldehydes and ketones **(Gandotra et al., 2012)**. The some studies showed a decreasing in ash content in fish during frozen storage which was related to the drip loss during thawing process **(Gandotra et al., 2012)**.

Table 2 showed that moisture content in fresh sample was affected brining process was decreased respectively (84.72% to 33.99%) with significant different (p <0.05), while protein, fat, ash and energy values was affected brining process were increased. The obtained results in this study were agreed with results **Aberoumand and Ziaei-Nejad, (2015)**. The effects of frying cooking technique on the proximate composition (moisture, protein, lipid, ash and carbohydrates) of the studied fish are presented in Table 3. Moisture content of raw and fried fish was 84.72 %, and 21.10% respectively. There was significant loss in the moisture content of raw fish due to the cooking process by frying (p <0.05). This observation agreed with **El Sherif, Ibrahim and Abou-Taleb, (2011)** for fried Tilapia fish as well as for some fish species **(Gokoglu, Yerlikaya and Cengiz, 2004)**. The protein content in raw the fish was significant increased (p <0.05) in the fried samples. Increasing protein content in the cooked fish samples (fried) due to the water loess during cooking. Protein contents were 7.67 % in raw and 14.16% fried, respectively. Similar data showed that deep-fried fish had the highest protein value comparing other cooking methods **(Gokoglu, Yerlikaya and Cengiz, 2004; Kucukgulmez et al., 2006)**. Data given in Table 3 showed that total lipids in raw and fried samples were 6.52, and 62.76%, respectively. These values were differing significantly (p <0.05). The higher lipid content of fried the fish due to the absorption of oil and losing moisture during frying process **(Saguy and Dana 2003)**. Ash contents were 0.99 and 1.97 in raw and fried fish, respectively (Table 3). The differences of ash content higher of fried is due to more loss of moisture took place during deep frying cooking method **(Kucukgulmez et al., 2006)**. The changes found in the chemical composition of fish cooked by frying were similar to several studies carried out in other fish species included Sea bass **(Gokoglu, Yerlikaya and Cengiz, 2004; Kucukgulmez et al., 2006; Marimuthu et al., 2012; Garcia Arias, Ailvarez, and Garcia Linares, 2003)**.

Table 4 showed that pH was found 6.18 in fresh sample and increased significantly (p <0.05) to the value 6.50 in 7 days frozen fish. There was comparatively slow increase in pH between fresh fillets during freezing periods. It was increased from 6.18 to 6.50. There was significant differences (p <0.05) between fresh fish and frozen fish that was frozen for 7 and 21 days. No significant difference between groups of fresh fish that was exposed to the different freezing periods. These results are in accordance with **Erkan and Ozden, (2008)** who stated that the increase was due to an increase in volatile bases from the decomposition of nitrogenous compounds by endogenous or microbial enzymes. Obemeata, Nnenna and Christopher, (2011**)** observed that the increase in pH was higher in the 4 °C stored sample of Tilapia, indicating that biochemical and microbial changes are occurring faster in 4 °C stored fish. **Pawar et al. (2013)** showed slightly increased pH in *Catla catla* from 6.50 to 6.79 when stored at chilled temperature (-2 to -4 °C). The change in pH of fish sample is usually good index for quality assessment. The increase in pH is caused by the enzymatic degradation of fish muscle.

An increase in protein content in processing methods was due to aggregation of proteins after the removal of water molecules present between proteins **(Ninawe and Ratnakumar, 2008)**. The increase in method time and loss of moisture leads to denaturation of the protein **(Begum et al., 2013)**. The fat content increases in brining method with a decrease in water content as the lipid plus water content of fish flesh equal to 80% **(Bligh et al., 1988)**. The higher ash content was due to the substantial loss of moisture **(Mustapha et al., 2014)**. During brining, the mass transfer occurs basically between salt and water: the fish fillets takes up salt and loses water **(Chaijan, 2011; Oliveira, et al. 2012)**. Nutritional composition, such as protein, lipid, and ash, were increased due to the loss of water in fish fillets in the brining process **(Brás and Costa, 2010; Chaijan, 2011)**.

The increased protein degradation is occurred by major changes in the protein fraction of the salted fish and the increased NaCl concentration. Consequently, the decrease pH value is because of the increase of the ionic strength of the solution inside of the cells **(Goulas and Kontominas, 2005; Leroi and Joffraud, 2000)**. The decrease in sample lipid content during freezing was associated to the fat hydrolysis and due to the oxidation rancidity. Similarly **Siddique et al. (2011**) found that total lipid content decreased during frozen storage of three species of *Puntius*. The decrease in ash content was associated to the drip loss during thawing process **(Roopma et al., 2013)**.

Table 1 Proximate composition (g.100g^{-1}) and caloric value of fresh and frozen fish (*Parastromateus niger*), on a dry basis.

Parastromateus niger	Proximate composition					
	Moisture	Protein	Lipids	Ashes	Calories (Kcal.100g^{-1})	Calories (KJ.100g^{-1})
Fresh	84.72 ±0.27^{a}	7.67 ±0.32^{a}	6.52 ±0.21^{a}	0.99 ±0.07^{a}	89.36 ±1.7^{a}	373.52
Frozen 7 days	84.74 ±0.7^{b}	8.52 ±0.96^{b}	5.63 ±0.52^{b}	1.11 ±0.11^{b}	84.70 ±1.51^{b}	354.05
Frozen 21 days	74.65 ±0.3^{b}	18.64 ±0.92^{b}	5.48 ±0.42^{b}	1.23 ±0.12^{b}	123.88 ±1.44^{b}	517.82
Frozen 35 days	74.18 ±0.4^{b}	20.89 ±0.89^{b}	3.77 ±0.41^{b}	1.16 ±0.11^{b}	117.49 ±1.34^{b}	491.11

Note: Mean of samples analyzed in duplicate. The same letters in the column do not differ from each other at the 5% level of significance.

Table 2 Proximate composition (g.100g^{-1}) and caloric value of fresh and brined fish **(***Parastromateus niger***)** on a dry basis.

Parastromateus niger	Proximate composition					
	Moisture	Protein	Lipids	Ashes	Calories (Kcal.100g^{-1})	Calories (KJ.100g^{-1})
Fresh	84.72 ±0.27^{a}	7. 67 ±0.32^{a}	6.52 ±0.21^{a}	0.99 ±0.07^{a}	89.36 ±1.7^{a}	373.52
Brined	33.99^{b}	21.45^{b}	17.35^{b}	27.21^{b}	241.95^{b}	1011.35^{b}

Note: Mean of samples analyzed in duplicate. The same letters in the column do not differ from each other at the 5% level of significance.

Table 3 Proximate composition (g.100g^{-1}) and caloric value of fresh and fried fish Parastromateus niger, on a dry basis.

Parastromateus niger	Proximate composition					
	Moisture	Protein	Lipids	Ashes	Calories (Kcal.100g^{-1})	Calories (KJ.100g^{-1})
Fresh	84.72 ±0.27^{a}	7.67 ±0.32^{a}	6.52 ±0.21^{a}	0.99 ±0.07^{a}	89.36 ±1.7^{a}	373.52
Fried	21.10^{b}	14.16^{b}	62.76^{b}	1.97^{b}	262.75^{b}	1098.3^{b}

Note: Mean of samples analyzed in duplicate. The same letters in the column do not differ from each other at the 5% level of significance.

Table 4 pH value of raw, brined and frozen 7 days, 21 days and 35 days fish samples *Parastromateus niger*.

	Raw	Brined	Frozen 7 days	Frozen 21 days	Frozen 35 days
pH	6.18^{a}	6.05^{b}	6.50^{c}	6.23^{d}	6.06^{b}

Note: Mean of samples analyzed in duplicate. The same letters in the row do not differ from each other at the 5% level of significance.

CONCLUSION

Proximate composition and energetic values of *Parastromateus niger* fish showed significantly ($p \leq 0.05$) differences between brined, cooked and raw samples and during frozen storage. The moisture loss in the boiled and fried samples reached its highest level. Also, protein, fat and energy were relatively high. The nutritional value of fish *Parastromateus niger* retained until the end of the storage period. Therefore, it must be beyond the process of useful and optimized processes that can lead to the production of superior products with nutrients, beyond consumer satisfaction. To prevent the impact of processing on fish nutritional and physical and chemical constituents, the use of appropriate methods for fish processing and fish products was essential.

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EFFECT OF FUZZY-CONTROLLED SLOW FREEZING ON PUMPKIN (*CUCURBITA MOSCHATA* DUCH) CELL DISINTEGRATION AND PHENOLICS

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ABSTRACT
Freezing has been widely used to preserve vegetables including seasonal pumpkins. This work aimed to investigate the effects of freezing on pumpkin cell disintegration and phenolics. A fuzzy logic control (FLC) system was built to obtain better temperature control of the freezing system. Changes in cellular disintegration, electrical conductivity and phenolics content were evaluated. The angle measure technique and principal component analysis were used to delineate the surface texture changes of the frozen pumpkin cells. The results showed that FLC offered reliable temperature control performance. Freezing at -18 °C for 7 h caused the highest cell degradation of 0.467 on the disintegration scale. Decomposition was also indicated by an almost double increase in electrical conductivity. The changes in texture were accurately reflected in the mean angle spectra and 81.3% and 7.4% of the variability due to treatments could be explained by two principal components respectively. Freezing pumpkin at -18 °C for 6 h correlated to the maximum increase in total phenolics of 70.44%. The increased phenolics were dominated by caffeic acid, chlorogenic acid and p-coumaric acid. In conclusion, as the freezing system exhibits positive effects on the phenolics content of pumpkin, it may be employed to process seasonal pumpkin to obtain higher value from the produce.

Keywords: pumpkin; freezing; fuzzy; disintegration; phenolic

INTRODUCTION
Pumpkins can be cultivated in warm areas around the world **(Kumar, Rattan and Samnotra, 2016)**, are cheap to grow and provide a high nutrient content **(Provesi and Amante, 2015)**. The valued nutrients of pumpkin include phenolic compounds ranging from 4.44 to 5.65 mg GAE/g dry matter **(Mendelova, et al., 2017)**, flavonoids (4.4 mg/100 g) and anthocyanins (0.14 mg/100 g) **(Oloyede, et al., 2012)**.

The most frequently used preservation method for fresh food from living tissue or cellular food materials is freezing **(Li, Zhu and Sun, 2018)**. Freezing causes plant cells to undergo cellular disintegration related to dehydration and mechanical damage mechanisms. These allow cells to lose water by diffusion, leading to irreversible shrinkage until the cells collapse **(Fellows, 2009)**. The migration of intracellular water to form ice crystals in the extracellular area results in intracellular dehydration and an increase in the ionic strength of the cell. The mechanical damage to cells results from membrane distortion and stress on rigid structures **(Zaritzky, 2011)**. Freezing of plant foods enhances the release of phytochemicals due to the extraction effects induced during the process **(Leong and Oey, 2012)**.

The effect of processing on food structure can be precisely assessed by imaging of the food surface. Within the food image, information about the surface of food and cells is stored as an array of pixels of different intensities to form the image texture **(Quevedo, et al., 2002)**. Using the angle measure technique (AMT), a raw image is converted to a one-dimensional signal, then a number of points are selected along the signal point and the mean angle (MA) at each selected point is calculated. This step is repeated to reach the last point, to generate an MA spectrum of the image. The MA spectra obtained from images of various treatments are then used for principal component analysis (PCA) to obtain image separation **(Fongaro and Kvaal, 2013)**.

There is scarce information on the effect of slow freezing on the cellular integrity and phenolics content of pumpkin. Based on our preliminary study, temperature controls of the currently available household freezers employ a conventional thermostat with which a precise freezing temperature is difficult to achieve. Among other control systems, fuzzy logic control (FLC) is regarded as an updated approach which does not require a precise model to build and would improve refrigeration efficiency **(Saleh and Aly, 2015)**. Furthermore, FLC is considered an intelligent control system that well accommodates the implementation of heuristic knowledge of a system **(Jantzen, 2013)**. This study therefore aimed to elaborate the

effect of fuzzy-controlled slow freezing on pumpkin cellular structures and phenolic compounds.

Scientific hypothesis

Fuzzy-controlled freezing increases pumpkin cell disintegration and phenolics content.

MATERIAL AND METHODOLOGY

Pumpkin samples

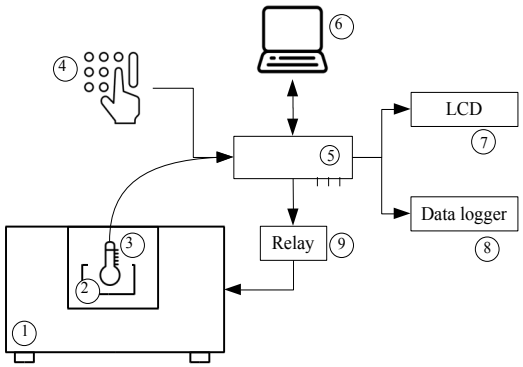
Pumpkin fruits (*Cucurbita moschata* Duch) were obtained from a local market. Confirmation of the correct species was obtained from an expert at the Materia Medica government herbal centre. The fruits were chosen based on the following conditions: fresh, clean, correct maturity stage, free from apparent physical defect and biological spoilage, and weighing approximately between 2.5 and 4.0 kg each. The fruits were kept under controlled conditions within the recommended temperature range of 7 – 13 °C **(Raju, Chauhan and Bawa, 2011)** and RH of 50 – 70% **(Maynard and Hochmuth, 2007)**. A data logger (Extech RHT20, FLIR Systems, Inc., Massachusetts, USA) was used to monitor the humidity and temperature. The actual temperature and RH range during experiments were 10.3 – 10.8 °C and 48 – 68.4% respectively. The fruit exocarp, fibrous strand and seeds were removed, and the pulp was obtained by cutting the fruit following the rib.

For freezing experiments, samples were cut into 2 cm x 2 cm x 3 cm pieces weighing 250 g. For the cell damage experiments, the pumpkins were prepared in the form of a cylinder 1 cm in diameter and 3 cm in length obtained using a stainless steel laboratory borer. Dried pumpkin powder for determining total phenolics content and liquid chromatograph mass spectrometer (LCMS) analyses was prepared by slicing of frozen samples after complete thawing for 2 h at room temperature. The samples were then dried at 55 °C in a hot air-drying oven (FDH6, Maxindo, Indonesia) for 24 h. The samples were finally ground (Philips HR2116, Indonesia) and sieved (Retsch 5657, Germany) at mesh 50 to obtain uniform particles with a maximum size of 300 μm. The powdered samples were vacuum-sealed and stored in a dark container at room temperature for analysis.

Development of fuzzy-controlled freezer

A new household chest freezer (Sansio SAN-153F, China) was used to build the fuzzy-controlled freezing system. The freezer was an air-stagnant type designed for a tropical climate with a capacity of 153 L, R134a cooling medium and cyclopentane insulation. A microcontroller-based FLC was developed to control the temperature and freezing time during the experiment. The design of the freezing system used in the experiment is presented in Figure 1.

The FLC was designed to operate based on two input variables, namely temperature error and delta error, and pulse width modulation (PWM) as the output variable. The temperature error was obtained from the difference between temperature setting and the temperature of food samples. Temperature measurement was carried out by inserting a three-wire WZP-187 PT100 sensor (accurate to ±0.01 °C) into the samples placed on the hanging basket inside the freezer compartment in approximately the geometric centre.



Note: 1. chest freezer, 2. pumpkin sample, 3. temperature sensor, 4. key input, 5. microcontroller, 6. computer, 7. LCD monitor, 8. data logger, 9. solid state relay.

Figure 1 Design of the fuzzy-controlled freezing system.

The temperature difference between two consecutive measurements was assigned to the delta error variable. The input variables were then fuzzified based on their type and range which were determined based on trial-and-error experiments in the initial FLC development. The variables and degree of membership (μ) used to develop the FLC are presented in Figure 2.

The Takagi–Sugeno–Kang method was used during the defuzzification step to obtain PWM values based on 18 rules (Table 1) to produce output values which were then directed to the freezer compressor. A Crydom solid state relay D2450-10 with attached ULN2803A integrated circuit was employed to control the inductive load of the relay. The temperature was read at 1 s intervals and logged using a Deek Robot logging shield.

A computer program was written to implement the developed system and uploaded onto an ATmega2560 Arduino microcontroller using a personal computer. The fuzzy inference system modelling of MATLAB® R2017a was used to help design and simulate the fuzzy logic system. The difference between the PWM output of the modelled and actual systems was expressed as the mean absolute percentage error. Before experiments, the PT100 sensor was calibrated by comparing the resistance of the sensor and the value on the data sheet using 30 temperature points ranging

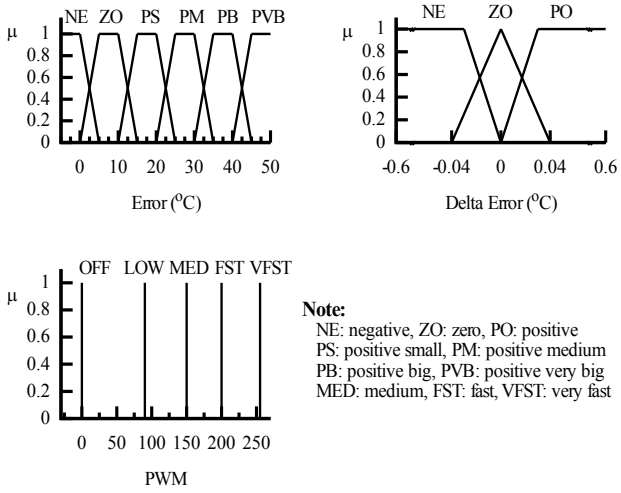


Figure 2 Variables of the fuzzy-controlled freezing system.

Table 1 Rule base of the control system.

Error	Delta error		
	Negative	Zero	Positive
Negative	Off	Off	Off
Zero	Low	Medium	Medium
Positive small	Low	Medium	Medium
Positive medium	Medium	Medium	Fast
Positive big	Medium	Fast	Fast
Positive very big	Fast	Very fast	Very fast

from 3 to -15 °C. The developed system was eventually calibrated using a Fluke 724 Calibrator (Fluke Corporation, USA) and method AS 2853:1986 at the Central Laboratory of Science and Engineering, University of Brawijaya, Indonesia.

Freezing experiments

The effects of freezing on pumpkin tissue were investigated at temperatures starting from -3 °C for 4 to 8 h. The starting freezing temperatures for the experiments were determined based on the initial freezing point of pumpkin which was ascertained during pre-experiments using the cooling curve method **(Rahman, et al., 2009)**. For this purpose, the empty freezer was run at a medium temperature setting to reach a stable temperature for at least 2 h. Then, the temperature sensor was inserted into a 200 g sample approximately at its centre to approach the coolest point. The sample was then placed on a 15 mm thick Styrofoam pad in the freezer compartment and allowed to freeze **(Bainy, Corazza and Lenzi, 2015)**. The changes in temperature were recorded on a personal computer and plotted using Gnuplot version 5.2. The process was stopped when the temperature reached the end freezing point. Based on the logged data, a cooling curve was drawn, then the initial freezing and end freezing temperatures were determined based on the zero slope of the starting plateau and end of the plateau on the curve respectively. The freezing rate was determined based on the time required by the coolest sample area to decrease from 0 to -18 °C **(Pham, 2014)**.

Cell disintegration experiments

The effect of treatment on pumpkin cell damage was estimated by measuring the electrical conductivity (EC) of samples with stirring using a CON 700 meter (Oakton Instruments, Singapore). The cell disintegration index Z was calculated using the formula $Z = (\sigma - \sigma_i)/(\sigma_d - \sigma_i)$, where σ is the EC of the treated sample, and σ_i and σ_d refer to the conductivity of intact and maximally damaged cellular samples respectively **(Vorobiev and Lebovka, 2009)**. The latter value was obtained from the EC of thermally processed samples (85 °C, 15 min) to represent the maximum disintegration of the samples **(Maskooki and Eshtiaghi, 2012)**. The samples (1.0 cm in diameter and 3 cm in length) were vacuum-packed in an embossed polyethylene bag to avoid leaching **(Leong and Oey, 2012)** and immersed in hot water at 60 and 80 °C for 15 min. The EC measurements were carried out at a controlled room temperature of 22 °C. The prepared samples were immersed in 100 mL of distilled water in a beaker after the samples reached room temperature.

Microstructure analysis

Samples for microstructure analysis were prepared using the air-drying preparation method **(Pathan, Bond and Gaskin, 2010)**. Pumpkin samples of 20 mm x 15 mm were cut to approximately 1 mm thick using a B Braun 22 scalpel and allowed to dry slowly at room temperature for 12 – 24 h. Images of the prepared samples were then acquired using a scanning electron microscope (SEM) (FEI™ Inspect S50, USA). Before imaging, the dried samples were sputter-coated (Emitech SC7620 Sputter Coater, UK) with a thin layer of gold–palladium (approximately 10 nm, 5 mA, 180 s) at room temperature and a pressure of 10^{-2} Pa. The samples were observed at an acceleration voltage of 15 kV, and difference magnifications were used to obtain images.

Images obtained from the SEM machine were saved at a resolution of 34 pixels per cm (34 bits per pixel) in tagged image file format (TIFF). The complexities of image textures were characterized using the AMT method **(Kvaal, et al., 2008)**. The image processing was carried out using ImageJ software version 1.52p **(Schneider, Rasband and Eliceiri, 2012)** with AMT plugin. The setting values for ATM algorithms were maximum scale 500 pixels, method of unfolding spiral, unfolded pixels start from 0, and 500 sampling points.

Total phenolics content (TPC)

Approximately 0.5 g of dried pumpkin samples was added to 20 mL of 95% ethanol and macerated for 24 h in the absence of light at room temperature. The extracts were then filtered through a Whatman filter paper and stored in a brown bottle before analysis. TPC was estimated using the Folin–Ciocalteu method **(Alara, Abdurahman and Ukaegbu, 2018)**. The TPC in the pumpkin samples was calculated based on the standard curve of gallic acid constructed over a concentration range of 0 to 50 mg/L. The resulting regression line was y = 0.0044x + 0.093; R^2 = 0.9876, where y was the absorbance at 765 nm and x was the concentration from the calibration points. Results were reported as gallic acid equivalent in milligrams per gram of dried samples (mg GAE/g dw).

LCMS analysis

LCMS analyses were performed on untreated pumpkin and samples frozen at -18 °C for 6 h which exhibited the highest TPC. The TPC extracts were further filtered (0.45 μm) and analysed **(Zdunic, et al., 2016)**. The analyses were carried out using a Shimadzu LCMS - 8040 LC/MS equipped with a Shimadzu column Shim Pack FC-ODS (2 mm D x 150 mm, 3 μm) (Shimadzu Corporation, Japan), and run with the following settings: injection volume 1 μL; capillary voltage 3.0 kV; column temperature 35 °C; flow gradient 0/0 at 0 min, 15/85 at 5 min, 20/80 at 20 min and 90/10 at 24 min; flow rate 0.5 mL/min; sampling cone 23.0 V; MS ion type [M]+; collision energy 5.0 V; desolvation gas flow of 6 L/h and temperature of 350 °C; low energy CID fragmentation; ESI ionization with scanning of 0.6 s/scan (*m/z*: 10 – 1000); source temperature 100 °C; and 80 min run time. Ethanol (95%) and water were used as solvents. Curve areas on chromatograms of untreated and treated samples were then compared to determine the individual phenolic changes due to freezing.

Statistical analysis

The average means of the response variables were compared using a one-way analysis of variance test. Pearson correlation was used to determine correlation between the disintegration index and EC of samples. PCA was run to identify the variability of surface texture due to freezing. All statistical tests were carried out using the R software environment for statistical computing, version 3.6.2 **(R Core Team, 2018)** at $p < 0.05$.

RESULTS AND DISCUSSION

FLC performance

The system calibration report showed that the temporal and spatial variations of the freezer were within acceptable values of -1.0 and 1.9 °C respectively. During the performance test at a setting temperature of -10 °C, the freezer demonstrated an average rise time of 21.38 ±2.40 min, settling time of 31.36 ±1.29 min, and steady error positive and negative of 1.55 ±0.11 and -1.28 ±0.33 °C respectively. The small temperature errors could be used as evidence for the high performance of fuzzy-controlled refrigeration systems **(Cheung and Kamal, 1997)**. Other related data that support the system's robustness include the strong correlation between actual and theoretical resistance values ($y = 1.0001x - 0.0211$; $R^2 = 0.9996$) and the small mean absolute percentage error value of the system (9.52%).

Based on the freezing rate of pumpkin samples in the experiment (4.2 °C/h) as shown in Table 2, the developed freezing system can be classified as slow freezing. This process is typically characterized by a freezing rate of 1 – 10 °C/h **(Brown, 1991)**. The determination of this freezing rate is important to ensure the correct slow rate applied in the experiment. The initial freezing point of pumpkin samples was -3.06 °C (Table 2). This point is close to freezing temperature of similar vegetables (-0.8 to -2.8 °C) **(Fellows, 2009)**. Differences in the water content and other chemical compounds in the sample and the values used in the literature may have been responsible for the disparities. The observed freezing point of pumpkin was then used as the basis for further freezing experiments to have a correct starting point for temperature assignments for the rest of the experiments.

Table 2 Freezing parameters of pumpkin.

Run number	Freezing point (°C)	End point of freezing (°C)	Time to reach from 0 to -18 °C (min)	Rate of freezing (°C/min)
1	-3.04	-6.09	219	0.08
2	-3.07	-7.60	242	0.07
3	-3.17	-6.62	299	0.06
4	-2.87	-6.02	279	0.06
5	-3.14	-8.53	273	0.07
M	-3.06	-6.97	262.40	0.07
SD	0.12	1.08	31.73	0.01

Cell disintegration

The fuzzy-controlled freezing of pumpkin caused great cell disintegration (Figure 3). There was a tendency for decomposition to increase as the temperature was lowered for a longer process time. This was notable particularly for freezing of less than 7 h and prolonged treatments would eventually have decreased the disintegration. The maximum disintegration of 0.467 ±0.051, as a result of freezing at -18 °C for 7 h, corresponded to an increase of EC from 36.2 µS/cm for untreated samples to 74.75 µS/cm. This ionic elevation could be caused by intracellular water which migrates extracellularly to form ice crystals during the freezing process **(Zaritzky, 2011)** and the presence of damaged cells **(Vorobiev and Lebovka, 2009)**. This result is consistent with other work by **Marra (2013)** which showed that slow freezing increases the EC of potato, carrot and apple, attributable to changes in the structure of the food samples used. Furthermore, that work also indicated a greater increase for quick freezing.

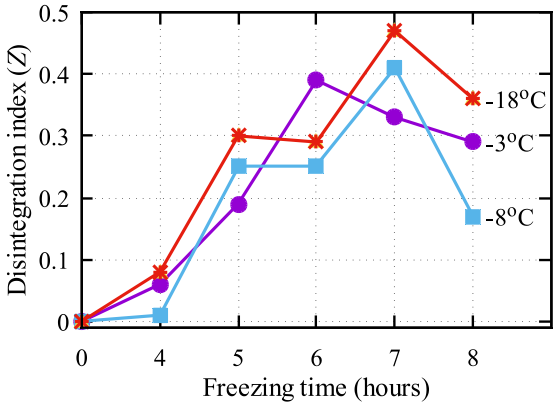


Figure 3 Effect of freezing on disintegration of pumpkin cells.

The degree of cell disruption in the present study could be considered sufficient with respect to heat-sensitive nutrient retention. More severe cell degradation may be achieved by applying a higher temperature during the thermal processing of samples, but this could destroy heat-sensitive polyphenols. **Moreira et al. (2019)** have shown that boiling and steaming of pumpkin accounts for 23.41 − 44.63% and 24.39 − 43.00% losses of total phenolic compounds respectively. Epimerization of polyphenols may occur under high-temperature processing; therefore, low-temperature processing and storage are preferred **(Deng, et al., 2018)**.

The cell disintegration and EC of samples was positively correlated (Pearson's $r = 0.95$; $p < 0.001$). This indicates that EC could be used for rapid assessment of pumpkin cell disintegration during freezing storage. Several studies have also suggested the use of EC to assess cell degradation due to food processing of potato, carrot, apple and sugar beet **(Marra, 2013; Maskooki and Eshtiaghi, 2012)**.

Microstructure changes

Untreated pumpkin tissue consisted of cells which are closely bound together by the cellular structure, roughly circular in shape and notably different in size (Figure 4a). This characteristic of visual structure is similar to the result reported in other work **(Rojas and Augusto, 2018)**. The disintegration of cells after freezing may be conventionally concluded by comparing the images for frozen samples (Figure 4b) and their untreated counterparts. The frozen

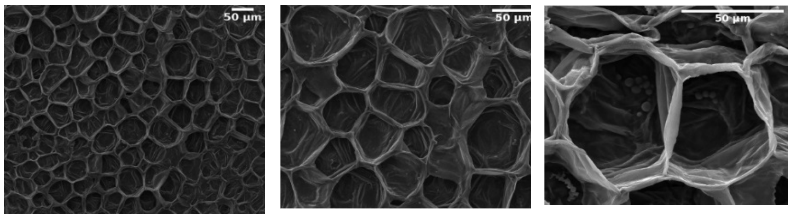


Figure 4a Microstructure of intact, untreated pumpkin cells at different magnifications, from left to right 500x, 1000x and 2.500x; scale bar 50 µm.

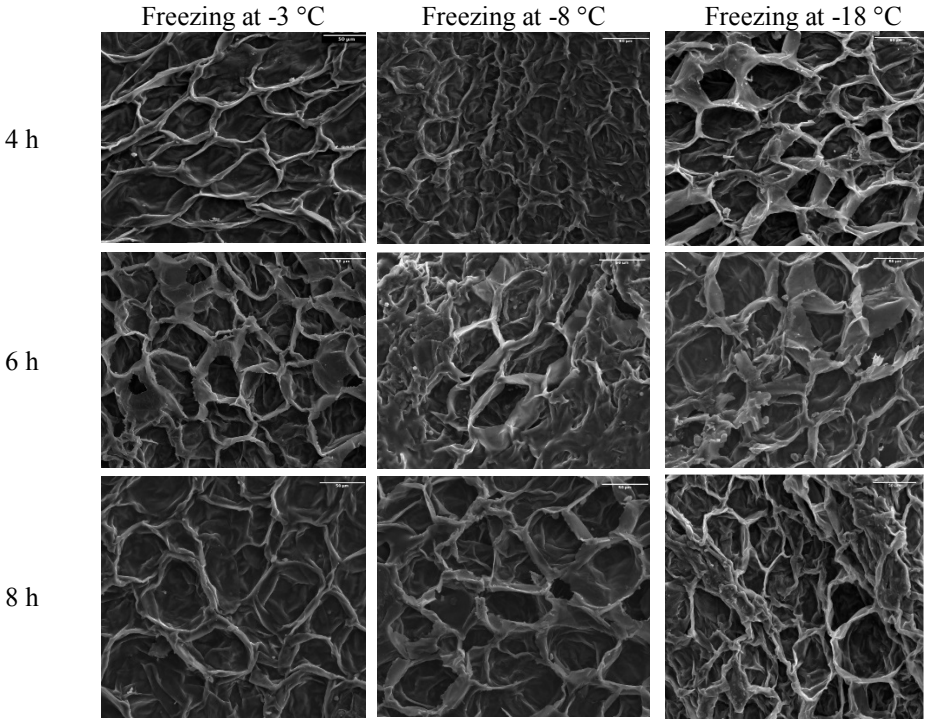


Figure 4b Comparison of microstructure of pumpkin samples after treatment at different temperature (°C) and duration of freezing (h); magnification 1000x, scale bar 50 µm.

tissue became distorted, some cells undergoing rupture and suffering from an increase in irregularity in shape. The cellular disintegration of plant tissue could be a result of water losses from the cell which lead to cell shrinkage **(Fellows, 2009)**. Membrane distortion and stress on rigid cell structures due to ice crystal growth can also cause mechanical damage to cells **(Zaritzky, 2011)**. Generally, the main factors affecting cellular structure during freezing are the formation of ice crystals, migration of water, and the intrinsic characteristics of the cells **(Li, Zhu and Sun, 2018)**.

In the present study, AMT was used to delineate and to conform the changes in surface texture of pumpkin samples as a response to freezing treatment. As the microstructure undergoes changes, the surface texture is expected to change accordingly. Along with other factors such as the illumination and imaging system used during experiments, the surface texture of samples is a determinant characteristic of the visual texture **(Kvaal, et al., 2008)**. Based on the MA spectra obtained, it was evidenced that frozen pumpkin exhibited a higher MA compared to untreated pumpkin (Figure 5). Increases in MA in the MA spectrum correspond to the irregularity, coarseness and intricacy of surfaces **(Huang and Esbensen, 2000)**. The rough texture could be the result of surface texture changes which reflect cellular decomposition following the freezing treatments. Therefore, it is clear that the surface texture of the samples deviated from that of untreated pumpkin, indicating the effects of the treatments.

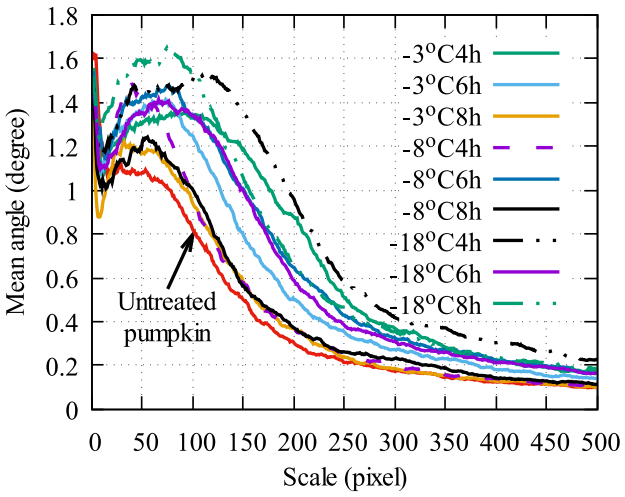


Figure 5 Mean angle spectra of pumpkins after freezing; a higher mean angle value indicates rougher surface texture.

The PCA results provide more specific information on the variation of texture as a result of various freezing treatments. The majority of the variability of sample texture in the projection model can be addressed by two principal components, accounting for 88.2% and 7.4% of variability respectively (Figure 6). The result clearly shows that untreated samples contribute a small degree of variability to the data set as signalled by the AMT spectra. The results also indicate that samples frozen at -18 °C have clearly

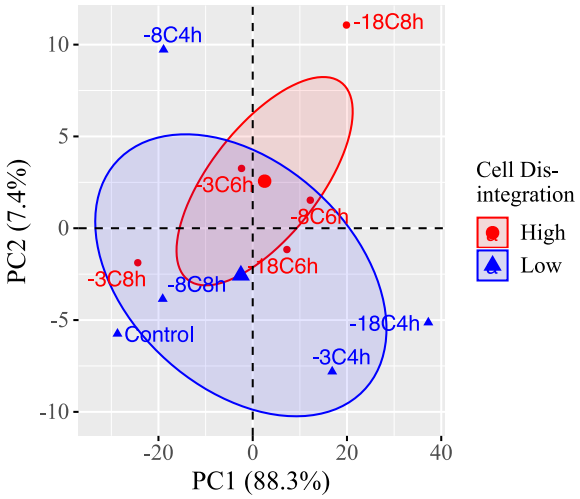


Figure 6 PCA result for pumpkin samples from different freezing treatments.

different texture characteristics compared to those frozen at -8 or -3 °C as they are separated on the PCA graph. Frozen samples which have undergone more severe cell disintegration seem to separate apart from the rest. This could indicate the correlation between cell disintegration and the texture of pumpkins due to freezing. A similar correlation has been demonstrated on red sweet pepper tissue using high hydrostatic pressure and pasteurization treatments **(Hernández-Carrión, et al., 2015)**. Due to the apparent complexity of the image textures, the MA spectra and valuable PCA outputs would substantially increase the accuracy in drawing conclusions compared to solely visual analysis of the sample images.

TPC

The TPC of untreated pumpkin was 8.49 ±0.08 mg GAE/g which is higher than the range of 4.44 – 5.65 mg GAE/g db **(Mendelova, et al., 2017)** reported previously. This may be attributable to differences in variety, environment and agricultural practices. The freezing treatments substantially increased the TPC of pumpkin samples, ranging from 9.02 ±0.03 to 14.47 ±0.05 mg GAE/g. The most notable increase resulted from freezing at -18 °C for 6 h (Figure 7). The effect of lowering temperature on the TPC increase was statistically significant (p <0.05). Although the freezing time appeared to increase the TPC, the effect was not statistically significant (p >0.05).

The increase in TPC can be attributed to the extraction effect of slow freezing on food **(Leong and Oey, 2012)**. The maximum gain of TPC in the present research (70.44%) is higher compared to other findings. The increase in TPC of strawberries frozen in an air-stagnant household freezer at -

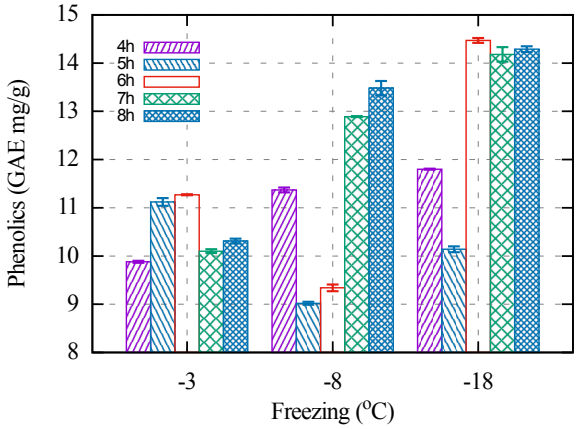


Figure 7 Total phenolics content of frozen pumpkins.

Note: Different notations within values in respective group indicate difference values at p <0.001.

27 °C for 1 week reported by **Bulut et al. (2018)** is 3.01%. An increase in TPC of 27.34% has been also reported for cagita fruit following freezing at -40 °C **(Santos, et al., 2018)** and of 17.15% for 'Hudson' spinach after 3 months of freezing **(Bystrická, et al., 2015)**. The extent to which these increases vary could be related to different influencing factors including the food matrix **(Leong and Oey, 2012)** and process parameters used in the experiments. The use of FLC to achieve better control of temperature is an interesting independent variable to which attention may be drawn. The scarcity of published data on the type of freezer temperature controller and on performance tests before systems are used in experiments makes comparison of any results difficult to attain. A precise freezing temperature of a desired point for experimental purposes is difficult to achieve when an air-stagnant household freezer with an analogue thermostat controller is employed. As **Cheung and Kamal (1997)** showed that the performance of a refrigeration system controlled by fuzzy logic is better than that controlled by a proportional derivative method, the small temperature errors observed during the freezer performance test in the present research could serve as evidence for better temperature control or process performance.

LCMS results

The LCMS test showed that slow freezing increased phenolic compounds, primarily caffeic acid, chlorogenic acid and p-coumaric acid (Table 3). Significant increases in other phenolics such as isorhamnetin-3-O-rutinoside, ferulic acid and naringenin were also detected. The increase in phenolic compounds such as anthocyanins and flavonol glycosides due to the extractability effect of freezing has also been demonstrated for grapes **(Tomaz, et al., 2017)**. LCMS provides individual changes in phenolics which cannot be observed by Folin–Ciocalteu assay alone. LCMS tests are also more precise. Ascorbic acid in pumpkin flour samples may act as a reducing agent and interfere with the Folin–Ciocalteu assay, hence reducing test specificity **(Sánchez-Rangel, et al., 2013)**. Dried pumpkin samples used for LCMS analysis may contain ascorbic acid as revealed by related work that pumpkin powder produced by fluidized drying still contains as much as 21.16 mg vitamin C/100 g **(Khan, et al., 2019)**.

Table 3 Increase in pumpkin phenolics due to freezing as detected by LCMS.

Retention time (min)	Compound	Formula	Exact mass	Curve area increase
1.84	p-Coumaric acid	$C_9H_8O_3$	164.0473	1158.7066
3.28	Esculetin	$C_9H_6O_4$	178.0266	839.7814
4.64	Caffeic acid	$C_9H_8O_4$	180.0423	1374.3696
5.01	Scopoletin	$C_{10}H_8O_4$	192.0423	928.1558
5.04	Ferulic acid	$C_{10}H_{10}O_4$	194.0579	1059.2264
9.73	Naringenin	$C_{15}H_{12}O_5$	272.0685	1054.8360
10.32	Kaempferol	$C_{15}H_{10}O_6$	286.0477	774.9496
10.50	Catechin	$C_{15}H_{14}O_6$	290.0790	468.7586
12.42	Chlorogenic acid	$C_{16}H_{18}O_9$	354.0951	1220.7587
14.43	Kaempferol 3-arabinoside	$C_{20}H_{18}O_{10}$	418.0900	884.5015
21.39	Vitexin	$C_{21}H_{20}O_{10}$	432.1056	502.27583
21.43	Kaempferol-3-O-rhamnoside	$C_{21}H_{19}O_{10}$	431.0984	793.8315
22.62	Kaempferol-3-O-D glucoside	$C_{21}H_{20}O_{11}$	448.1006	869.5909
23.19	Astilbin	$C_{21}H_{22}O_{11}$	450.1162	164.3881
24.02	Isoquercitrin	$C_{21}H_{20}O_{12}$	464.0955	929.7765
36.85	Isorhamnetin-3-O-rutinoside	$C_{28}H_{32}O_{16}$	624.1690	1059.9496
37.06	Rhamnazin 3-rutinoside	$C_{29}H_{34}O_{16}$	638.1847	879.9009
46.91	Isorhamnetin 3 rutinoside 4' rhamnoside	$C_{34}H_{42}O_{20}$	770.2269	979.3314

CONCLUSION

In the present study, a slow freezing system controlled by a fuzzy logic algorithm was developed to study pumpkin cell disintegration. Freezing causes severe cell degradation which can be clearly observed by an increase in disintegration index and surface texture changes. Frozen pumpkins exhibit rougher texture or a higher MA at the respective scale as compared to untreated samples. As a result of cell damage, increases in EC and phenolic compounds are also detected. The maximum increase in TPC of 70.44% resulted from freezing at -18 °C for 6 h. The increased phenolics compounds are mainly caffeic acid, chlorogenic acid and p-coumaric acid. Information obtained from cell damage, texture analysis and phenolics content are all in agreement to support the favourable effects of fuzzy-controlled slow freezing on phenolics from pumpkin.

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EVALUATION OF THE PHYSIOLOGICAL STATE OF FEIJOA (*FEIJOA SELLOWIANA* BERG) IN SUBTROPICAL RUSSIA

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ABSTRACT

The article presents the results of research examining varietal diversity with respect the activity of oxidative enzymes (EC 1.11.1.6) and the dry matter and Proline accumulation of leaves under optimal and stressful conditions. For feijoa, the most stressful period in the subtropics of Russia, with respect to hydrothermal conditions, occurs between July and September. Studies have shown that the highest degree of enzymatic activity is observed in August in the 'Superba' variety of feijoa, which was used as a control in this study, and the lowest level of activity was observed in the 'Sentjabrskaja' variety. The long-term water deficit experienced in September coincides with fruiting in feijoa. This causes a change in catalase activity in leaves, which is maintained until it is inhibited. Form ShV-1 of feijoa is characterised by its metabolic stability. In fact, the activity of oxidative enzymes in leaves of the variety is stable. Dry matter content per unit area increases as the leaf grows. During the drought period, which coincides with active fruiting, the leaves of the 'Dachnaja' variety and the ShV-1 form accumulate significantly less dry matter than other varieties. In the 'Dagomysskaja' variety, the intensity of organic matter consumption via respiration and outflow exceeds visible photosynthesis, which is expressed as a negative value (average = 1.96 $g.dm^{-2}$ h). To fully characterise the physiological state of feijoa plants under the influence of abiotic factors and catalase activity in the humid subtropics of Russia, indicators of dry matter accumulation and true photosynthesis intensity can be used.

Keywords: feijoa; enzymes; catalase; photosynthesis; stability; stress factors

INTRODUCTION

According to the modern Botanical classification, feijoa belongs to the *Myrtaceae* family of the order Myrtales **(Bose, Mitra and Sanyal, 2001; Omarova and Kulyan, 2019; Ryndin, 2019)**. The family includes 72 genera and about 100 species that grow in the tropics and subtropics, mainly throughout America, North-West Africa and Australia. Feijoa is native to the subtropical zone of South America. In its wild form, it grows over a large area consisting of shrubby and mixed forests in southern Brazil, Paraguay, Uruguay, and Northern Argentina. These areas lack sharp fluctuations in air temperature and precipitation **(Belous, Omarov and Omarova, 2014; Kedelidze et al., 2015; Phan et al., 2019)**. The genus Feijoa includes three species, *F. obovata* Berg, *F. shenkiana*, and *F. sellowiana* Berg. In culture, *F. obovata* and *F. shenkiana* do not occur. Since its use is advantageous relative to the other varieties of feijoa, only *F. sellowiana* (synonyms: *Acca sellowiana* Berg, *Orthostemon sellowiana* Berg) is widely known and industrially significant.

Feijoa Zellova (*F. sellowiana*) is an evergreen tree/shrub that grows to a height of 3 to 5 meters or more. The crown of a young plant is compact, but with age, usually after entering the fruiting season, it spreads out. Before fruiting, bushes tend to increase in height. Afterward, apical shoots slow growth, and side shoots contribute to increases in the diameter of each plant. When grown industrially, different forms of feijoa plants are used that consist of erect plants with a pronounced stem, vigorously growing types that are structurally similar to trees, squat types with dense branching and a low-growing, compact and leafy type.

Feijoa is one of the most valuable crops, since its fruits are made up of 11.4 – 12.4% dry substances, 7.92 – 9.16% total sugars, 1.56 – 1.84% sucrose, 6.20 – 7.41% invert sugars, 1.60 – 2.45% glucose and fructose 3.36 – 5.24%. The dry weight of feijoa is also, 0.94 – 1.43% acids, producing a sugar acid index is of 6.09 – 9.06 units. Other components include vitamin C (26.0 – 32.4 mg), starch (0.92 – 1.16%), pectin (1.28 – 1.91%) and hemicellulose (3.63 – 4.25%) **(Bontempo et al., 2007; Belous, Omarov and Omarova, 2014; Aoyama, Sakagami and Hatano, 2018; Kedelidze et al., 2015; Montoro et al., 2020)**.

Plants of different varieties can be characterised by their metabolic features and considering what type of physiological and biochemical processes occur in a plant can facilitate the elucidation of adaptation mechanisms that are used to maximise plant fitness under changing environmental conditions. The purpose of our study was to examine metabolic characteristics of a number of varieties

and forms of feijoa. For example, the main indicators of the water status, and features of enzymatic activity, characteristics of the pigment apparatus and accumulation of free Proline were assessed. These indicators were selected because the photosynthetic apparatus is highly sensitive to environmental factors, and the content of plastid pigment has traditionally been used as a diagnostic indicator of the plant health **(Belous, Klemeshova and Malyarovskaya, 2018; Horčinová Sedláčková et al., 2018)**.

The enzymatic activity of catalase (CAT), an oxidative enzyme, varies in plants as they react to adverse factors **(Hodges et al., 1997; Apel and Hirt, 2004; Belous, 2012; Abilfazova and Belous, 2018)**, and the accumulation of damage can inactivate the enzyme. The strength of CAT inhibition depends on many factors (plant age, varietal characteristics, etc.). The accumulation of free Proline occurs as a response to various damaging factors, since Proline helps to reduce the magnitude of damage **(Nilsen and Orcutt, 1996)**. Finally, the intensity index of true photosynthesis is used to characterise overall photosynthesis, and is directly related to the functional state of the plant. The intensity of true photosynthesis is associated with the accumulation of assimilates within the plant **(Osmond et al., 1987; Gaspar et al., 2002)** and can be calculated by assessing changes in the amount of dry matter within leaves over a defined period.

In this article, we have assessed CAT activity in feijoa leaves, which is a diagnostic characteristic of the functional state of the plant. Also, we have determined levels of protein and dry matter accumulation, and have measured the intensity of true photosynthesis, which determines assimilation levels within the plants assessed.

Scientific hypothesis

Determination of CAT activity is a marker of the adaptive potential of plants and can be used to identify crop resistance to hydrothermal stress.

Active defense mechanisms in feijoa plants allow plant leaves to accumulate a large amount of dry matter.

Evaluation Proline accumulation in leaf is necessary for fully characterizes the physiological status of feijoa plants.

MATERIAL AND METHODOLOGY

Plants used in the study were a one-zoned ‘Superba’ variety of feijoa, three varieties from the institution (‘Dagomysskaja', 'Dachnaja’ and 'Sentjabrskaja’) and well as a promising (ShV-1), which was grown in the collection garden of the Russian Research Institute of Floriculture and Subtropical Crops. The determination of physiological parameters was carried out at the laboratory of plant physiology and biochemistry of the Institute. Leaf samples consisting of 52 – 60 leaf pieces were collected dynamically during both the most optimal and highly stressful dry growing season. Experiments were conducted using three field and three laboratory replicates.

The analysis of CAT activity in leaves was performed using by gasometrical method **(Tretyakov, 1990)**. The principle of the method for determining CAT activity is based measuring the quantity of oxygen released throughout the decomposition of hydrogen peroxide. CAT activity was expressed in mL oxygen released per g of raw plant tissue.

Percentage dry substance values were calculated by drying samples at a temperature of 105 °C **(Erzhanov, 2016)**. The process is designed to remove both hygroscopic and external moisture. To calculate the content of components within the dry substance, the mass of the determined component was expressed as a percentage of the mass of the dry sample.

The intensity of true photosynthesis was calculated based on measured quantities of accumulated dry matter **(Marakaev, 2005)**. The intensity of true photosynthesis is equal to the sum of the intensity of visible photosynthesis and the intensity of the consumption of organic substances for respiration and outflow to other organs (since the fluctuation of water in the leaf masks change in dry matter mass, leaves were cut when fully saturated with water).

To determine free Proline content, the Bates method was used **(Bates, Waldren and Teare, 1973)**. The method is based on the interaction between free proline and ninhydrin reagent, which can be measured colourimetrically as a pink-red colour is produced. Free Proline content was determined using a calibration curve constructed using Proline solutions ranging from 50 to 150 mgL^{-1}, and values were expressed in micrograms Proline per 1 g of raw mass.

Statistical analysis

Statistical processing of experimental data was carried out using the ANOVA package in STATGRAPHICS Centurion XV (version 15.1.02, StatPoint Technologies) and MS Excel 2007. Statistical analyses included univariate analysis of variance, which is a method for comparing averages using variance analysis and a t-test) and variance analysis (ANOVA). Differences between means compared using least significant difference (LSD) were considered significant when $p < 0.05$. All experiments were performed in triplicate and values were expressed as mean ± standard deviation (SD). Differences between the samples were assessed using unpaired t-tests.

RESULTS AND DISCUSSION

The resistance of plants to adverse environmental factors is largely determined by the activation of the antioxidant enzyme system, which inhibit the damaging effects of oxidative stress. CAT is a key enzyme involved in protection against oxidants, and the enzyme catalyzes a number of metabolic reactions. In particular, it catalyzes the decomposition of hydrogen peroxide to water and molecular oxygen. CAT activity is a marker of the adaptive potential of plants and can be used to identify the resistance of crops to hydrothermal stress, thus establishing their adaptive potential **(Belous, 2012; Apel and Hirt, 2004; Hodges et al., 1997)**.

The effect of factors such as drought on plants includes the suppression of many physiological processes. Simultaneously, drought stress activates protective mechanisms. In recent years there has been a significant increase in temperature throughout the summer period during which has prolonged drought periods for active vegetation. There ideal conditions were determined to study the effect of hydrothermal stressors on the metabolic

activities of subtropical crops, in particular, feijoa. If May precipitation of 70 – 100 mm exceeds the norm (on average 110 mm), then in other periods will experience a stable water deficit. Precipitation is reduced from 82% (June) to 21 – 26% (August) of the average annual precipitation rate. This is accompanied by increased air temperatures, which reach daytime maximum values of 34.1 – 37.0 °C, which is 4 – 9 °C higher than long-term parameters. This causes not only early attenuation of growth processes, but also fruiting in many crops, which affects the crop quality and quantity.

Changes in enzymatic activities were measured during a period of peak hydrothermal stress, which occurred as the crop was fruiting. According to a number of authors, when plants get into water deficit conditions, there is an increased regulation of certain AOS-producing enzymes (for example, catalase, ascorbate peroxidase, guaiacol peroxidase, etc.) **(Arbind, Sheela Devi and Sundareswaran, 2014; Hodges et al., 1997)**. An increase in the activity of a number of enzymes during the progression of water deficiency in plants has shown that a direct consequence of water deficiency is damage to the cell membrane. The results highlight the important role of certain antioxidant enzymes and compounds in protecting against drought stress **(Sofo, Dichio and Xiloyann, 2010)**. Our studies have shown that, in general, the enzymatic activity of CAT ranges from 104.4 to 170.4 mL O2.g^{-1} of raw weight. The highest CAT index is expected in August (the most stressful period of vegetation) was observed in the 'Superba' variety, which is a control, and the lowest activity observed was determined using the 'Sentjabrskaja' variety (Figure 1). Long-term water deficiency provoked a change in the CAT activity of feijoa leaves. This was especially true in September, when plants were in the fruit loading phase, which enhanced plant stress in nearly all varieties. This was reflected by the inhibition of CAT activity, which was observed in all varieties with the exception of the 'Sentjabrskaja', whose enzymatic activity was 1.3 times higher in September than its annual average value. It must be noted that form ShV-1 did not experience noticeable changes in CAT activity, which suggests that metabolic processes on the form are stable, despite changes observed regarding the strength and duration stress experienced.

The dynamics of dry matter accumulation in feijoa leaves revealed that dry matter content per unit area increased with leaf area (Figure 2). Water stress can limit the accumulation of dry matter in the leaves, so it is important to select varieties resistant to water deficiency **(de Lacerda et al., 2003; Apel and Hirt, 2004; Amirjani, 2010; Boussadia, Ben Hassine and Braham, 2018)**. Moreover, during the drought period, which coincided with the processes of active fruiting, leaves of the 'Dachnaja' variety and the ShV-1 form accumulated dry matter significantly less than other varieties. The most active protective mechanisms observed were in plants of the 'Sentjabrskaja' variety. The continuing drought allowed leaves, despite the active formation of fruits, to accumulate greater quantities of dry matter than other varieties ('Sentiabraskaja' averaged up to 53.90 g dry matter, while other varieties averaged 49.85 – 52.91 g).

Proline levels in the leaves of varieties and forms in July ranged from 93.75 to 201.79 mg.g^{-1}, and depend on the genotypic characteristics of plants (Figure 3). Throughout the period of high stress (August), we observed a 1.5 – 2.8 fold increase in Proline levels in feijoa leaves. The greatest numbers observed were determined in 'Dachnaja', 'Dagomysskaja' varieties and form ShV-1 (266 – 270 mg.g^{-1}). Low concentrations of Prolin in 'Sentjabrskaja' and 'Superba' varieties confirmed their resistance to abiotic stressors. These data in which Proline levels increasing under stressful conditions is consistent with studies by other authors **(de Lacerda et al., 2003; Ashraf and Foolad, 2007; Amirjani, 2010)**, who reported that increasing Proline content is a common physiological response to drought, mineral nutrition deficiency, and other adverse effects in plants. Determination of free Proline content can serve as an express method for determining the level of plant resistance, which will allow at the early stages of the selection process to reject less than stable breeding material **(Bates, Waldren and Teare, 1973; Nilsen and Orcutt, 1996; Amirjani, 2010)**.

The activity of photosynthetic processes, determined by measuring increases in the amount of dry matter present within cells, is a key indicator of plant resistance, since the active accumulation of photosynthetic products in conditions of stress is associated with high levels of plant resistance **(Marakaev, 2005, Horčinová Sedláčková et al., 2018)**. Calculations of the intensity of true photosynthesis during this period confirmed the physiological state of feijoa plants. Thus, the 'Superba' variety (4.82 – 13.56 g.dm^{-2} h dry matter) is characterised as being highly resistant to stress. It is known that not all dry matter that is formed accumulates in leaves. Some is consumed in the process of respiration. Thus, in the 'Dagomysskaja' variety, the intensity of organic matter consumption for respiration and outflow exceeded visible photosynthesis, which was expressed as a negative average value (-1.96 g.dm^{-2} h). The result was in accordance with the small difference observed in the dynamics of dry matter accumulation of the variety (Figure 2).

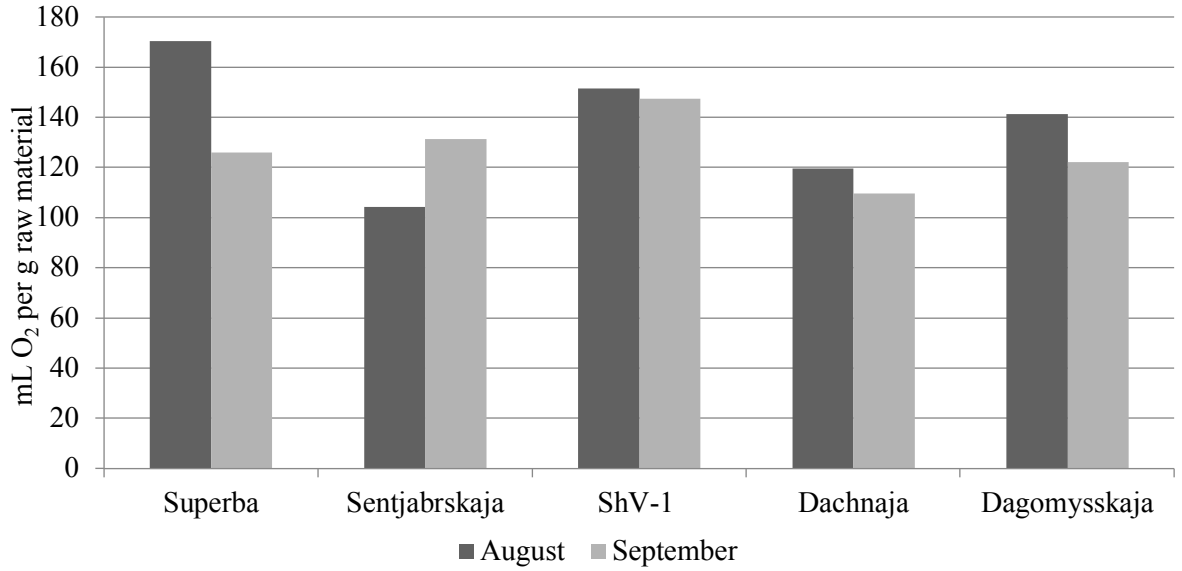

Figure 1 Catalase activity in feijoa leaves. Note: LSD ($p \leq 0.05$) = 12.16 (August) and 5.54* (September); * – NS.

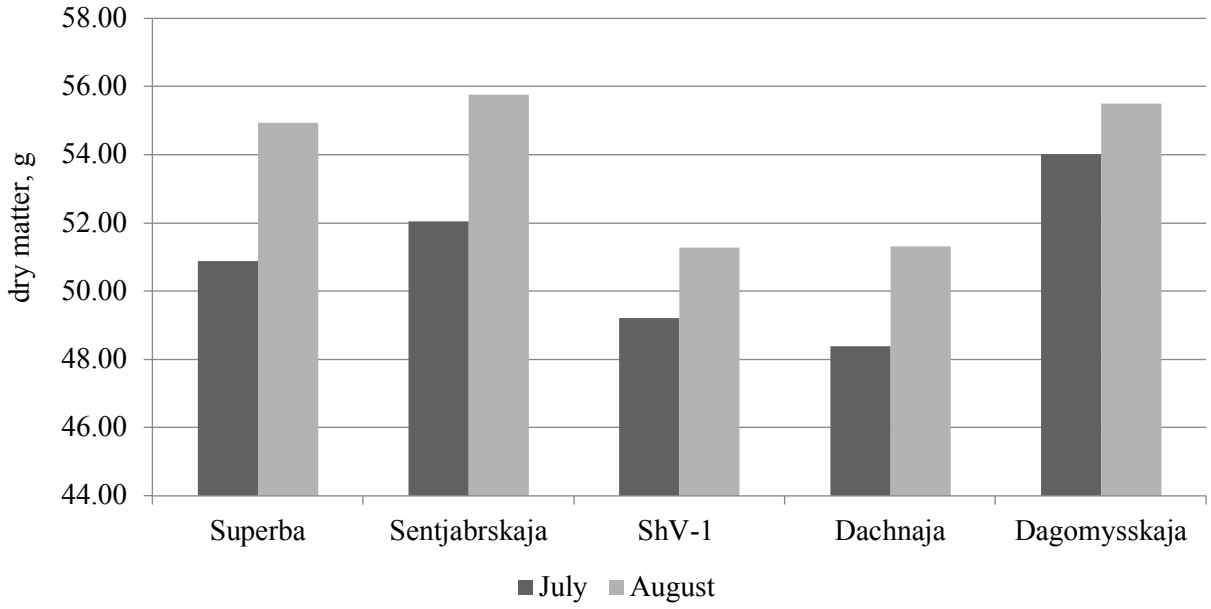

Figure 2 Dynamics of dry matter accumulation (%) in feijoa leaves. Note: LSD ($p \leq 0.05$) = 3.02 (August) and 2.15 (September); all differences were significant.

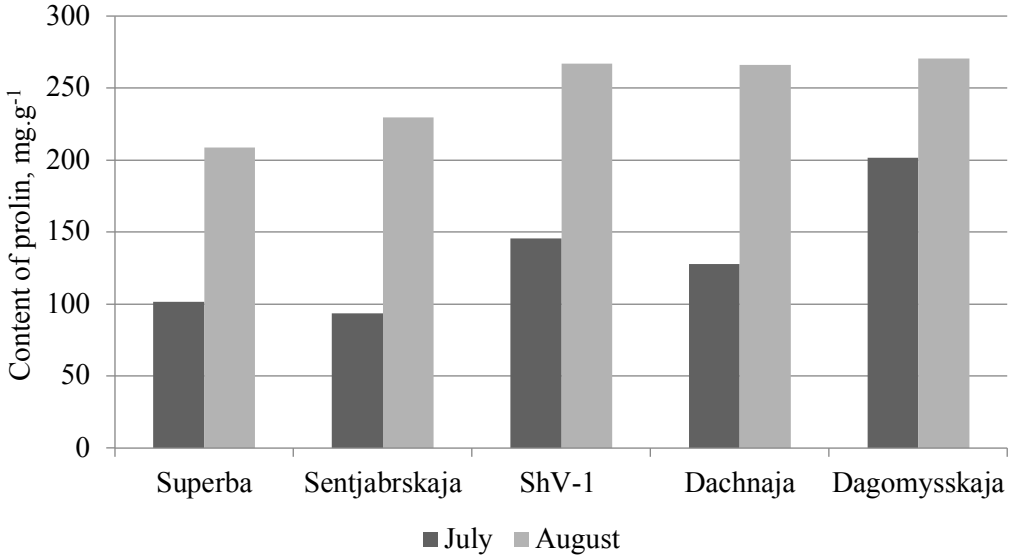

Figure 3 Dynamics of Proline accumulation (mg.g^{-1}) in feijoa leaves. Note: LSD ($p \leq 0.05$), NS.

CONCLUSION

The study has shown that varieties and forms of feijoa subjected to stress factors (drought and high air temperatures during active vegetation) altered CAT activities in their leaves. It should be noted that form ShV-1 did not significantly alter CAT activity indicators as its stress level increased, which indicates that it is a form that is highly resistant to the stress factor. To fully characterise the physiological state of feijoa plants under the influence of abiotic factors in the humid subtropics of Russia, in addition to the activity of the oxidative enzyme CAT, it is necessary to use indicators of dry matter accumulation and the intensity of true photosynthesis.

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THE EFFECT OF INFRARED DRYING TO THE MICROSTRUCTURAL STRUCTURE AND TEXTURE OF WHOLE DUKU INTACT SKIN BY MEANS OF SCANNING ELECTRON MICROSCOPY (SEM) TECHNIQUE

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ABSTRACT
The Infrared method has the potential to extend the shelf life of duku fruit by drying the duku's skin into "shell likeness". Duku's skin drying using infrared method could change the shape and characteristics of duku's skin which would significantly affect the length of fruit shelf life. The texture of duku's skin for the treatment of infrared emitter distance of 6 cm, temperature of 400 °C and exposure time of 80 seconds was increasing with the storage time which made the fruit inside the skin to experience a passive modified atmosphere and increase the shelf life of duku. The 3D visual depiction of the optimization result on drying process using infrared had the largest porosity and cavity value in the treatment of infrared emitter distance of 10 cm, temperature of 300 °C, and exposure time of 80 seconds. At the magnification of 2500 times, with a resolution of 10 μm, it was found that the porosity and thickness of the duku's void were greater than duku fruit without treatment. The result of the porosity also found that drying process with the infrared emitter distance of 6 cm at temperature of 400 °C, and exposure time of 80 seconds has more stable porosity (without collapsing) which confirmed the result found on the texture of the skin. The results of scanning electron microscopy analysis and 3D visual analysis confirmed the results of optimization that had previously performed in the drying process of duku fruit using infrared method.

Keywords: Infrared; drying; SEM Image; duku

INTRODUCTION
Electron microscopy is a method of using a beam of electron instead of light that interact with the atom in the sample. This interaction is in the form of sample reflectance which contain the information of the sample. One type of electron microscopy is called Scanning Electron Microscopy (SEM). The image produced by SEM is a result of a reflectance of heat, emission of low energy and high energy backscattered electrons, light emission which due to the beam of electron will contain the detail information about the properties of the surface of the sample. These signals were then converted into a very detailed image. SEM has been used to analyse the conditions of processing materials and food structures. Besides being used to analyse the material and structure of the food, SEM also provides more complete information about the structure of food materials by displaying three-dimensional imaging and could facilitate to obtain quantitative and qualitative information. SEM is used to visualize the structure of food materials because it combines several features of both light microscopy and transmission electron microscopy **(Aguilera et al., 2000; Falcone et al., 2006)**.

The study of the microstructure of food has become important part of food research because the structure of food could have an influence on nutritional value, rheology and some texture attributes **(Lyu et al., 2017)**. Food processing such as thermal and non-thermal processes could affect the structure and composition of the food **(Mercier et al., 2011; Lewicki and Pawlak, 2003)**. Thermal food processing could reduce the nutrition or the nutritional bioavailability, and cause damage to the chemical and the organoleptic properties. Some examples of thermal food processing technology is drying. Drying is one of the food processing methods that could prolong the shelf life or preserve grains, fruits, vegetables and food in all varieties. The quality of dried fruits depends on the conditions of the drying process. Infrared radiation has been used in drying food product. Infrared radiation has been widely implemented in the food process because it has several advantages including reducing water content in food, having low energy consumption and relatively short processing time, and could maintain and ensure the condition of product quality **(Pan et al., 2009)**. Infrared radiation could also inactivate pathogens in the material. Infrared radiation could preserve volatiles **(An et al., 2015)** inactivate bacteria, spore, yeast, and mold by

controlling some of the influential parameters such as power of infrared heater **(Hamanaka et al., 2000)**, sample temperature **(Sawai et al., 2003)**, wavelength and the target wavelength **(Krishnamurthy et al., 2008)**, sample thickness **(Sawai et al., 2000)**, and sample water content **(Hamanaka et al., 2006)**. Some drying processes with infrared have been widely used including for fruits and foods. The unique characteristics of infrared radiation is the heat from emitter energy only hit the surface of food ingredients in a short time without raising the temperature of the material **(Li and Pan, 2014a)**.

The uniqueness of infrared radiation could be used to dry the skin of whole intact duku fruit. The relatively short time infrared exposure in drying of the skin would make the water on the skin of the whole intact duku to evaporate and dry the skin. Skin drying would turn the skin of the whole intact duku to dry and in turn made it into the "shell likeness". This "shell likeness" of the duku skin would mimic the condition of the inner flesh of duku fruit into a state of passive modified atmosphere **(Rahmawati et al., 2018; Rahmawati et al., 2019** which was shown to increase the shelf life of duku **(Saputra and Pratama, 2013; Saputra and Pratama, 2018)**. During the process of forming "shell likeness" on duku's skin, the heat and mass transfer simultaneously were involved in this drying process. In these processes, the fruits would undergo some volume changes either by shrinkage due to moisture loss or by expansion due to gas generation or pore formation. This shrinkage or expansion process would result in the changes of porosity of the fruit **(Aydogdu et al., 2015)**. The measurement of porosity is needed to solve the transport process for these conditions **(Hayakawa and Futura, 1989; Witrowa-Rajchert and Rząca, 2009; Pongpichaiudom and Songsermpong, 2018)**. The changes in pore formation in food during the drying process could be used to predict the thermal conductivity, mass diffusivity, thermal diffusivity, and other transport properties of food **(Rahman, 2007; Mavroudis et al., 1998; Xiong et al., 2015; Vincent, 1989; Scanlon et al., 1998)**.

Textural and mechanical properties of food process had been shown to have a correlation to the porosity. Crispiness also was shown to have a high correlation to the food porosity. The higher the apparent density of the agglomerate of the lower porosity, the stronger the agglomerate. Besides of porosity, the binding forces or adhesion of particle (i.e., structure) also affect the mechanical strength **(Pietsch, 1999)**. The variation of porosity, size of pore and pore size distribution have significant effects on the textural characteristics of dried foods **(Huang and Clayton, 1990)**.

The objective of this study was to determine the effect of infrared drying on the microstructural structure (porosity) and texture of whole duku intact skin by means of Scanning Electron Microscopy (SEM) technique.

Scientific hypothesis

The hypothesis of this research was that the microstructural changes of intact duku's skin caused by the infrared drying could create a passive modified atmosphere within duku which was shown by the scanning electron microscopy

MATERIAL AND METHODOLOGY

Samples of Duku

Duku (*Lansium domesticum*) used in this study was bought from a local farm in the area of South Sumatra Province, Indonesia. Duku fruit selected based on the appearance of smooth yellowish colour of the skin without blemish, free from contamination of microorganism, and were selected in the diameter range of 2. 5 to 3. 5 cm. The fruits were then exposed to a pair of Infrared emitter (IRE) with the distance between the emitter at the top and bottom of 6 and 10 cm, the IRE temperature of 200, 300, and 400 °C, the exposure time of 50, 60, 70 and 80 seconds respectively according the treatment allocated by the experimental design. After exposing duku to the IRE, the fruit was stored in a showcase at 11 – 15 °C for 25 days. The physical and chemical properties of duku's were then measured periodically as described on previous study **(Rahmawati et al., 2018)**. The result of previous study on the effect of IRE distance, temperature, and time of exposure on the physical and chemical properties changes were then optimized to by means of using Response Surface Methodology **(Rahmawati et al., 2019)**. The skin of duku's from the result of the optimization were then peeled and its microstructure were determined by means of SEM to reconfirm the result of Response Surface Methodology.

Scanning Electron Microscopy (SEM) Analysis

Analysis of SEM was carried out by selecting the optimum treatment of IRE with the RSM method (Design Expert Program® Version 11, 2018). Duku's were peeled and then the skin was freeze dried. The freeze dried of duku's skin was then cut longitudinally and the skin texture was observed using the Scanning Electron Microscope (SEM, JEOL® serial number 6510 LA) with the magnification of 100 times, 500 times, and 2500 times, and the resolution of 100 μm, 50 μm, 10 μm and with the depth of field 10 mm and 11 mm. The process of taking pictures and sample composition with a SEM instrument were performed by placing and pasting the sample on the SEM specimen holder with the longitudinal cross section leading upward of the objective lens. The space of the specimen holder was then vacuumed to 10^{-6} torr to ensure that the SEM column was free of air molecules. SEM was operated with the standard operating parameters including, High Voltage (HV) = 25 kV; Spot Size (SS) = 50; Work Distance (WD)= ±50 μm.

Data from SEM analysis were then analysed by using the Mountain Map Program® version 7. Mountain map measured the peak and valley of the duku's skin microstructure and measured the void within the surface of duku's skin.

Statistical analysis

The analysis of variance (ANOVA) and optimization for this research was performed with the help of Statistical Package (SAS® version 9. 4). The statistical design was a split-split plot. The details of the design could be seen on the previous paper **(Rahmawati et al., 2018; Rahmawati et al., 2019)**.

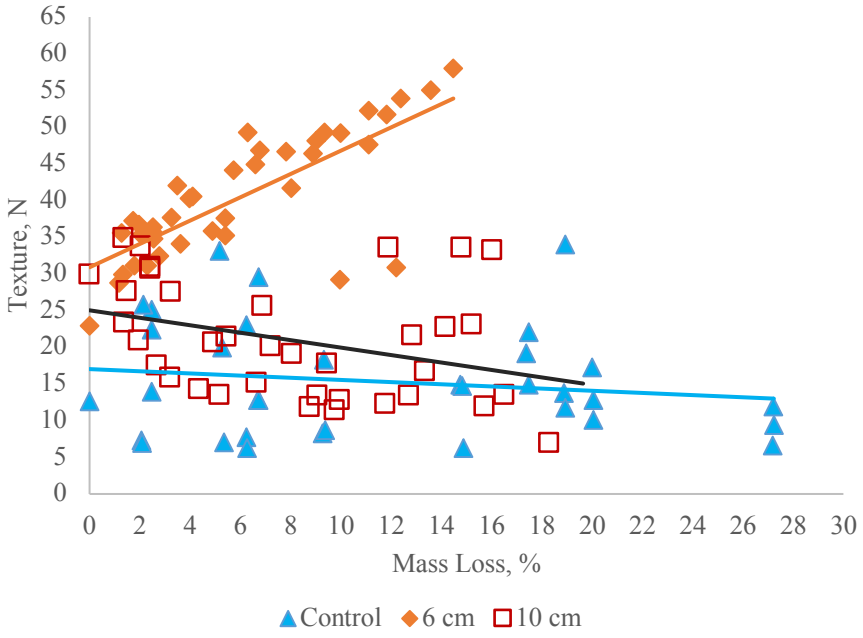


Figure 1 The correlation of duku's skin mass loss versus texture of duku.

RESULTS AND DISCUSSION

Mass loss on duku's skin and Fruit Firmness

Mass loss on the fruit skin, in general, had a correlation with the destruction of the skin due to deterioration. As expected for the plant product especially fruit and vegetables, the higher the mass loss the lower the texture. The lowering of the texture was due to the loss of liquid in the cell that would lower the turgor pressure of the cell which in turn would lower the texture. The lowering of texture for the control one was due to the deterioration or decaying of cells within the skin (Figure 1). However, in this study, the objective of the study was to dry the skin of duku which would make the skin turn into a cocoon likes and would make the fruit flesh inside the fruit to experience a passive modified atmosphere. So, the drying of the duku's skin would be expected to increase the texture. Unfortunately, for the treatment of IRE 10 cm, the correlation between the mass loss and the texture was a negative one. The texture of the skin did not increase with the increase of mass loss. Similar tendency to the treatment of IRE 10 cm was also shown by the control one. Initially, it was expected that the treatment of IRE at 10 cm would follow the hypotheses, however, even though duku's treated with infrared at IRE 10 cm showing a higher texture due to infrared drying but its texture had a similar tendency to the control. It seems the infrared drying treatment with IRE at 10 cm was not good enough in creating the passive modified atmosphere within the duku. It might be that the infrared radiation only hit the outermost of the skin but was not deep enough to penetrate the whole skin. This would make the surface of the skin dry but the inner part did not dried enough and the water from the inner part diffuse to the surface of the skin and this diffusion would make the surface of the skin became wet and lower the texture of the skin. It was observed that the wet skin on the IRE treatment of 10 cm start showing a decaying process.

Contrary to the result showing by duku on the infrared treatment of 10 cm and the control one, it was found that on this study the treatment of IRE at distance of 6 cm show a result as expected on the hypotheses which was showing that the higher the mass loss of the skin, the higher the texture. This tendency indicates that the increasing of the mass loss was due to the infrared drying process. The treatment of IRE of 6 cm seems dried the skin of duku deep enough and did not resulted in the diffusing of water from the inner part. The complete drying of the skin from the inner part to the outer part of the skin would result in harder shell of the skin which covered the duku's flesh. The increase hardness of duku's skin would in turn increase the texture of the skin and creates a cocoon or shell like which enclose the duku's flesh. This enclosure would create or mimics the environment of a passive modified atmosphere to the flesh of duku which would slow down the gas transmission to and out of the inside part of the duku as expected on the hypotheses. This condition confirms the result showed on the previous study **(Rahmawati et al., 2018**; and **Rahmawati et al., 2019)** which show that the treatment of infrared at IRE 6 cm gave an optimum result for infrared treatment of duku. Another measurement was performed to confirm this finding by means of inspection of duku's skin with Scanning Electron Microscopy (SEM).

SEM Analysis

Drying of fruit using infrared has been widespread **(Li and Pan, 2014a; Li and Pan 2014b; Pan et al., 2009; Ding et al., 2015; Léonard et al., 2008)**, but there was a limited literature which explains infrared drying for fruit skin without damaging the flesh of the fruit. The infrared drying process was widely used because it has high heat transfer coefficient, short processing time, and relatively low costs. Two aspects that were important for designing infrared heaters are the distribution of the spectrum and the energy or power intensity.

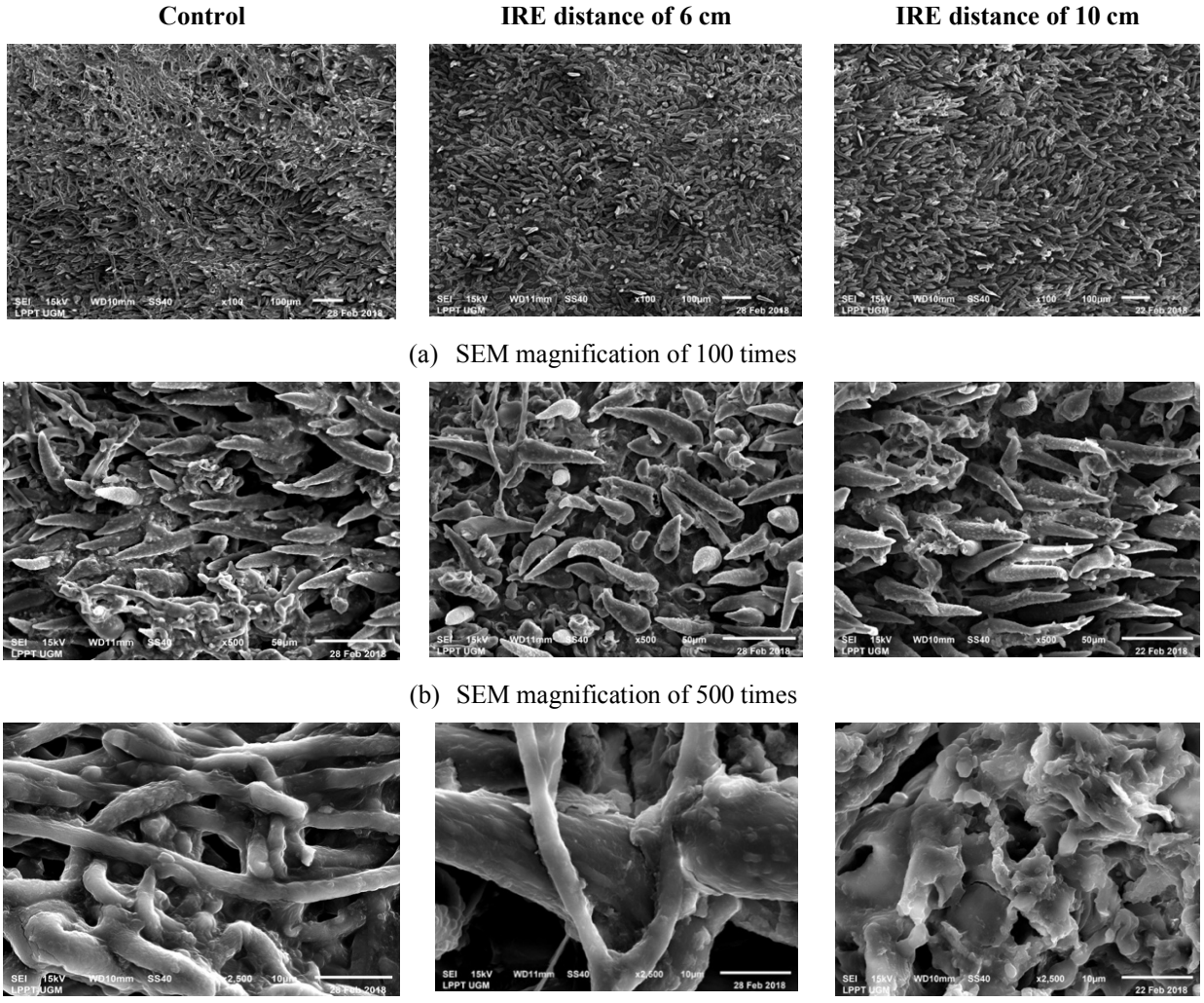


Figure 2 The Scanning Electron Microscopy of duku's skin.

The infrared heating spectrum needs to be considered because the energy which coming out of the emitter consists of different wavelengths, and the radiation fraction depends on a number of factors such as the temperature of the emitter, emissivity and others that would affect the texture of the fruit surface/heated material. Increasing infrared power would result in an increase in the drying rate. However, the faster drying rate would increase the process of mass transfer and might damage the fruit skin texture. Based on the result of optimization on previous study **(Rahmawati et al., 2019)** and the finding on Figure 1 about the effect of infrared drying to duku's skin, an attempt was performed to confirmed this finding by performing a Scanning Electron Microscopy (SEM). The microstructural observations of duku's skin was performed using the SEM method with the magnifications of 100, 500, and 2500 times, and the resolution of 50 μm, 10 μm respectively. The SEM was performed for the treatment at IRE 6 cm and IRE 10 cm, temperature of 400 °C, and exposure time of 80 seconds on the 14th days storage as the best treatment (Figure 2).

Visually, it could be seen the effect of infrared radiation to the structure of duku's skin. It could be seen that on the magnification of 500 times, the cell structure of duku's skin experiencing a change to the skin microstructure. The epidermal cells of the duku's skin undergoing an exfoliation in the cuticle part. The exfoliation of the cuticle could also be indicated that the waxy layer of the duku's skin has been destroyed by the heating process. Further observation on the magnification of 2500 times and the resolution of 10μm show that the skin of duku with IRE distance of 10 cm had some damage to the microstructure of its epidermal which was similar to the finding of condition of duku without magnification that duku's skin exposed to infrared radiation with IRE distance of 10 cm had deteriorated. A different result was found on the duku exposed to infrared with IRE distance of 6 cm. The microstructure of the skin, relatively, more intact and firmer which was due to a better texture of the skin show on the Figure 1.

However, even though it could be seen from Figure 2 that the microstructure of duku for the control one show a similar pattern with the one exposed to infrared radiation with IRE distance of 6 cm but its size was smaller compare the the fiber on the skin of duku exposed to IRE distance of 6 cm and 10 cm.

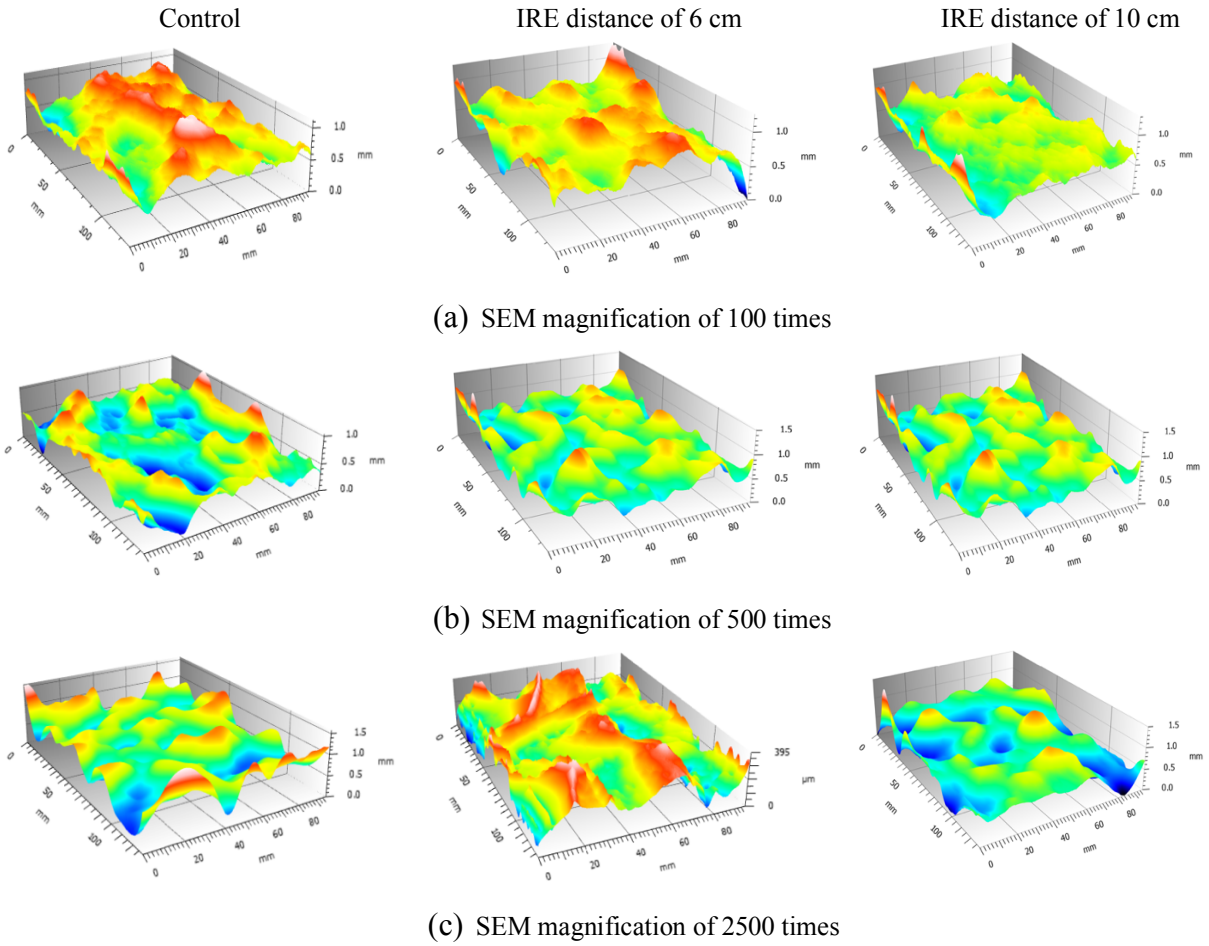


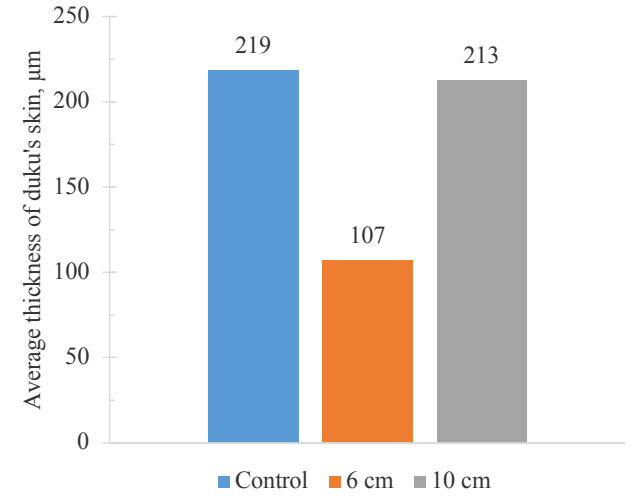
Figure 3 The 3D SEM countour plot of duku's skin processed by the Mountain Map Program.

The smaller fiber on the skin of duku for the control one turns out to be a mycelium of mold or fungus which grew on the deteriorated skin of duku. The control duku used on this experiment had started to deteriorate not just due to the loss of turgor pressure but also due to the mold which grew on the surface of the skin.

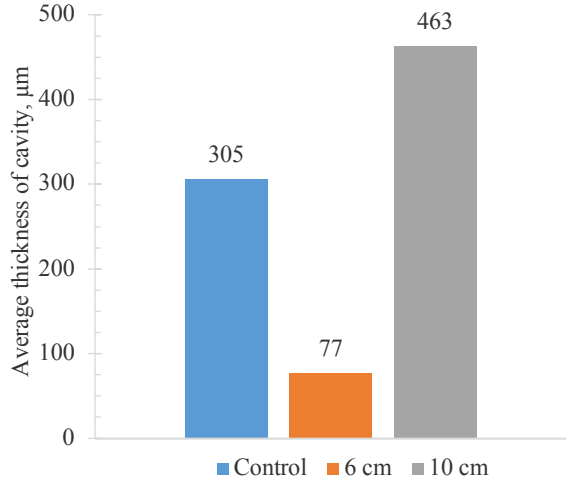
Another way to show the microstructure of duku's skin was by showing the SEM on a 3D picture. By using Mountain Map Program® a 3D picture of duku's skin as shown on Figure 3 were generated. This program besides creating the 3D picture also measured the height of the peak and the depth of the valley of the skin. This valley were the result of the skin which deteriorated due to uncomplete dry of the skin which resulted in the deeper valley of the skin. It was expected that that the fruit which underwent drying would have changes of pressure that modified its shape and deformed its skin **(Mayor and Sereno, 2004)**. This pressure difference would create more valley than the hills on the skin of duku.

At a glance, it could be seen that the infrared radiation with IRE distance of 6 cm, temperature of 400°C, and exposure time of 80 seconds, for the magnification of 100 times and 500 times produces more visual representation of red colour (hill) which indicates that the duku's skin exposed to infrared at IRE distance of 6 cm had a relatively intact dry skin. The hill on the figures show that duku's skin, relatively, still retain the skin layer and a stronger skin which simulate the passive modified atmosphere condition for the flesh of duku and increase its shelf life. However, a closure look at the magnification of 2500 times and resolution of 10 µm, show that even though the skin of duku for IRE distance of 6 cm had more hills compare to the control and duku exposed for IRE 10 cm but the unit and scale of the hills and valley was completely different. The unit for the control and IRE 10 cm were mm but the unit for the IRE 6 cm was µm. This finding indicates that the thickness of duku's skin exposed to IRE 6 cm had a thinner and compact skin compare to the control and IRE 10 cm. This finding also confirm the result showing on Figure 1 which show that the skin of duku exposed to IRE 6 cm had a thinner, stronger, and compact skin which would simulate the packaging of a passive modified atmosphere.

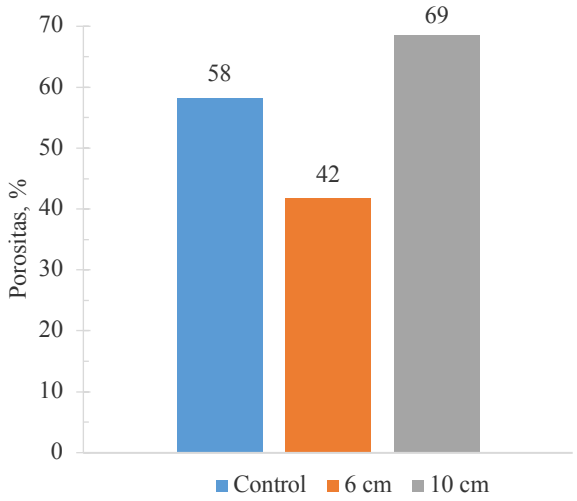
The porosity is the most important part that affects the strength of the agglomerate from the dried material. Porosity, pore size and pore size distribution have a significant influence on the texture characteristics of dry matter **(Huang and Clayton, 1990)**. The pores of the material could be characterized by the fraction of its porosity **(Rahman, 2007)**, shape of the pore, size of pores, number of pores, pore distribution, pore wall thickness, and number wall or face pores. Porosity could be obtained by comparing the volume of void with the total volume of material. Porosity values basically influenced by the overall drawing of SEM results in each magnification, resolution and depth of field values.



(a)



(b)



(c)

Figure 4 Average thickness (a), thickness of cavity (b), and porosity (c) of duku's skin for the control IRE 6 cm, and 10 cm.

The results of testing SEM with 2500 magnification, and 10 µm resolution show that the porosity and thickness of the duku's void without treatment was thicker than the exposure at 400 °C, 80 s at 6 cm distance of IRE. This was caused by the exposure process with IR has a solid and hard effect on the fruit skin, so the thickness of the void in the IR treatment was lower than the control treatment.

While the value of porosity and void thickness at the measurement of 2500 magnification, and 10 µm resolution at a distance of 10 cm has a higher value compared to the control, because during storage time the damage occurs on the texture besides that it could also be caused by the decay process.

A furthermore calculation of the peak and valley and the distribution of the area resulted on the average thickness of duku's skin, the average thickness of the void, and the porosity of the skin. The calculation shows that the thickness of duku's skin of 6 cm had the thinnest thickness (Figure 4 a). This result means duku's skin of IRE 6 cm is more compact than the duku's skin of IRE 10 cm and the control. The more compact skin would make duku's skin becoming a cocoon like and parallel to the result shown on Figure 1. A cocoon like skin would create an environment inside the skin like a passive modified atmosphere and prolong the shelf life duku.

The amount of porosity calculated for each SEM visual result would increase with the thickness of the voids **(Moon et al., 2014)**. The formation of pores could be divided into two types with inversion point and the other without inversion points. Factors that affecting the pore formation include internal and external factors. External factors include temperature, RH, pressure, gas atmosphere, and the radiation produced from dryers **(Rahman, 2007)**. From the statement it could be concluded that the biggest radiation produced at drying distance of 6 cm at 400 °C and exposure time 80 s has more stable porosity (without collapsing) when compared to drying with a distance of 10 cm at 300 °C and exposure time 80 s (Figure 4 b and 4 c).

CONCLUSION

The treatment of IRE 6 cm, temperature 400 °C and exposure time of 80 second increase the texture of duku's skin with the storage time. This increase made the fruit inside the skin to experience a passive modified atmosphere and increase the shelf life of duku. The 3D visual depiction of the optimization result on drying process using infrared had the largest porosity and cavity value in the treatment of 10 cm distance of IRE with 300 °C temperature of IR and 80s of exposure time. At the magnification of x2500, with a resolution of 10 µm, it was found that the porosity and thickness of the duku's void were greater than duku fruit without treatment. The result of the porosity also found that drying process with distance of 6 cm at 400 °C and exposure time 80 s has more stable porosity (without collapsing) which confirmed the result found on the texture of the skin. The results of SEM analysis and 3D visual analysis could confirm the results of optimization that had previously been done in the drying process of duku fruit using infrared method.

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EFFECT OF COMMERCIAL YOGURT STARTER CULTURES ON FERMENTATION PROCESS, TEXTURE AND SENSORIC PARAMETERS OF WHITE YOGURT

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ABSTRACT

In this work, we have compared and described the fermentation process of two commercial yogurt starter cultures during the white yogurt production. We used freeze-dried thermophilic starter culture YoFlex® YF - L812 and deep-frozen starter culture Delvo® Fresh YS – 241 for the production of white yogurts. We analysed titration acidity, active acidity, total viable counts, texture, and sensory parameters of white yogurts produced in laboratory conditions. This research was performed for dairy company Mliekareň Kopanice Selce, s.r.o., Slovakia. We did not found statistically significant differences (p >0.05) in titration acidity of both yogurts after 7 hours of fermentation. We did not found statistically significant differences (p >0.05) in the pH of both yogurts after 7 hours of fermentation. We found statistically significant differences (p <0.05) in all textural parameters (hardness, consistency, cohesion, and viscosity). The total viable count of microorganisms in yogurts after 24 hours of fermentation was 6.28×10^7 and 7.14×10^7 respectively.

Keywords: white yogurt; fermentation; titration acidity; texture; sensory parameters

INTRODUCTION

Yogurt is commercially produced through fermentation by lactic acid bacteria (commonly *Lactobacillus* spp. and *Streptococcus* spp.) at temperatures usually in the range of 27 to 40 °C **(Aguirre-Ezkauriatza et al., 2008)**.

Yogurt is an acid-milk product characterized by symbiotic cultures of *Lactobacillus delbrueckii* subs. *Blgaricus and Streptococcus thermophilus* and senanother *Lactobacillus* species. According to the Codex Alimentarius **(CA, 2003)** and legislation of the Slovak Republic, excess of living characteristic micro-organisms in acid-milk products like yogurt and yogurt with alternative culture has to be at least 10^7 CFU.g^{-1} and if the specific microorganism is presented in the label o the product then it has to be at least 10^6 CFU.g^{-1} **(MARDSR, 2016)**.

Yogurt is one of the most popular fermented dairy products, and its consumption is increasing worldwide **(Shiby and Mishra, 2013)**.

Its popularity is due to its sensory properties, health claims, and therapeutic effects. The most important sensory attributes of yogurt include texture, color, and flavor **(Sodini et al., 2004; Salvador and Fiszman)**.

Yogurt is typically characterized as a smooth, viscous gel with a characteristic taste of sharp acid and a green apple flavor **(Cheng, 2010)**.

Interaction of taste and odor with other sensory properties increases the complexity of the human perception of yogurt. The fat content, source of milk, and textural properties play an important role in the sensory properties of yogurt **(Routry and Mishra, 2011; Mende, Rohm and Jaros, 2015)**.

The decision to change the starter culture is very important and should be supported by information. The dairy must-have information on how the fermentation will take place.

This experiment was performed specifically for dairy company Mliekareň Kopanice Selce, s.r.o (Slovakia).

Scientific hypothesis

We are expecting the significant differences in TVC, physico-chemical, and sensory parameters of yogurt produced by two commercial starter cultures YoFlex® YF - L812 and Delvo® Fresh YS – 241.

MATERIAL AND METHODOLOGY

Chemicals

All chemicals used in the experiment were analytical grade and obtained by Centralchem (Slovakia).

Yogurt starter cultures

We used two thermophilic yogurt starter cultures in our experiment:

- **YoFlex® YF – L812**, Chr. Hansen Holding A/S
- **Delvo® Fresh YS – 241**, DSM Food Specialties.

Yogurt production

Yogurts were produced under sterile conditions in our microbiology laboratory of the Department of Food Hygiene and Safety at the Faculty of Biotechnology and Food Sciences of the Slovak University of Agriculture in Nitra.

We weighed a lyophilized thermophilic frozen culture on an analytical balance (Sartorius, Genius). To prepare 30 pieces of yogurt, we used 5 liters of full 3.5% pasteurized milk and 0.2360 g of starter culture (YoFlex® YF – L812) and 0.347 g of starter culture Delvo® Fresh YS – 241. The milk was heated to 40 °C and the starter culture was then added with gently stirring 5 min. When the culture dissolved, we poured the inoculated milk into the prepared sterile 150 mL plastic cups and covered them with aluminum foil to prevent contamination. The fermentation process was carried out in a thermostat (Thermo Fisher) at temperature 32 – 33 °C (YoFlex® YF-L812) and 40 °C (Delvo® Fresh YS – 241) for 48 hours.

The amount of weighed starter culture and the fermentation temperature were chosen according to the recommendations of the producers and requirements of the dairy company.

Consequently, samples of both yogurts were analyzed at hourly intervals for period of 48 hours.

Physical-chemical analysis

- Titration acidity was determined in duplicate by titration with 0.25 N NaOH factorised with 0.25 N oxalic acid, using 2 % phenolphthalein as an indicator. Titration of milk was carried out in titration bank and yogurt in a ceramic mortar. Results were expressed in °SH (Soxhlet-Henkel method) STN (1972). Slovak Technical Standard 570530. Methods for testing of milk and milk products. Article no. 58. – Determination of titration acidity according to the Soxhlet-Henkel.
- pH (SI Analytics Lab 845 pH meter milk before coagulation and Testo 206 – pH2 after milk coagulation were used). We used calibration standard solutions with pH 6.8 and 4.0.

Microbiological analysis

The total viable count was determined by the colony-count technique and expressed as (TVC.mL^{-1}). The plates were incubated for 72 h at 30 °C.

- PCA agar with milk powder (Biokar diagnostics). Composition: (1 L of medium): tryptone 5 g, yeast extract 2.5 g, glucose 1 g, skimmed milk powder (no inhibitory substances) 1 g, bacteriological agar 12 g, pH adjusted to 7 ±0.2.
- We have followed the recommendations of ISO 7889 (2003) *Yogurt. Enumeration of characteristic microorganisms.*

Textural analysis

Textural analysis of yogurts was performed after 24 hours of the fermentation process. We used:

- Texturomenter TA.XT Plus (Stable Micro Systems UK).
- Exponent software (Stable Micro Systems).
- Disc-shaped probe with back extrusion.
- The analysis of the textural properties of yogurt sample s was performed at 6 °C.
- The instrument was set up according to the manufacturer's recommendations: mode (a measurement of compression power), options (return to start), pre-test probe speed (1.0mm.s^{-1}), test probe speed (1.0mm.s^{-1}), return speed (10.0mm.s^{-1}), deep of probe penetration into the product (30 mm), Method of measurement (Auto – 10 g), reset mode (auto), frequency of data acquisition (400 pps), the test probe was cleaned after each measurement.

Sensory analysis

Sensory analysis was performed by a sensory panel consisting of twelve assessors (7 men, 5 women) after 24 hours of the fermentation process. The evaluators were first trained. The sensory panel analyzed the samples administered in the sensory laboratory.

- We used the scale and profile method.
- We used a hedonic scale where the value of 1 indicated that the quality of the yogurt was very poor and the value of 7 was excellent.
- We chose the classic profile, which consists of creating a rough profile, then reducing the descriptors were to eliminate hedonic terms (pleasant, tasty, good), quantitative terms (too much, weak, strong), inappropriate terms (salty for smell). We chose an intensity frequency from 0 to 5, where 0 was absent and 5 very strong. Subsequently, we fine-tune the fine profile, which consisted of an overall appearance, texture in the mouth, and taste.
- Then we set the polarity of the descriptors to positive and negative, which indicates the severity coefficient. The value for these coefficients is 1. We calculated the values using the pattern descriptor intensity x severity coefficient. We get both positive and negative numbers, from which we calculated the arithmetic mean and then visualized graphically.

Statistical analysis

Statistical analysis was performed in XLSTAT 2020.1 (Addinsoft).

- Physical-chemical and microbiological analysis: we used the Shapiro-Wilkov W test and t-test.
- Textural analysis: we used the Shapiro-Wilkov W test, F-test, t-test.

We considered the results to be statistically significantly different at the α 0.05.

RESULTS AND DISCUSSION

The development of titration acidity in white yogurts during the fermentation process is presented in the **Figure 1**. We did not found statistically significant differences (p >0.05) in titration acidity of both yogurts after 7 h of fermentation. A titration acid 24 °SH was reached after 7 h of fermentation and 29 °SH were reached after 24 hours of fermentation in both yogurts.

The results of pH development during the fermentation process are presented in **Figure 2**. We did not found statistically significant differences (p >0.05) in the pH of both white yogurts after 7 h of fermentation. The pH values were 4.71 (Delvo® Fresh YS-41) and 4.86 (YoFlex YF-L812). After 24 hours the pH values were 4.46 (YoFlex YF-L812) and 4.44 (Delvo® Fresh YS-41). When we compare

the values of pH, the highest decrease was noticed during the first 6 hours of the fermentation process and that trend continued until the final control point at 48 hours after fermentation. Our results are consistent with other authors **(Tamime and Robinson, 2000; Aguirre-Ezkauriatza et al., 2008; Tomovska, Gjorgievski and Makarijoski, 2016; Akgun, Yazici and Gulec, 2017)**. The pH of commercially available yogurt was 3.94-4.22 **(Matela, Pillai and Thamae, 2019)**. The fermentation process during industrial yogurt manufacture can be continuously controlled by online monitoring of pH, which offers a useful tool for integrated process control **(Soukoulis et al., 2007)**.

The inoculated milk began to coagulate during fermentation after 5 (Delvo® Fresh YS-41) and 6 hours (YoFlex YF-L812) and pH 4.4 was reached after 15 (Delvo® Fresh YS-41) and 16 hours (YoFlex YF-L812) of fermentation in cups.

The total viable count of microorganisms in yogurts after 24 hours of fermentation is presented in **Table 1**. TVC reaches the levels 6.28×10^7 CFU.g^{-1} (YoFlex YF-L812) and 7.14 CFU.g^{-1} (Delvo® Fresh YS-41) respectively. One of the essential characteristics of yogurt is that it must contain live microorganisms used for fermentation. The total amount of living characteristic microorganisms is characterized in the mentioned legislation and is quantified as the amount of colony-forming units (CFU), which for yogurts is equal to 10^7 CFU per gram of product. In both white yogurts, the legislation limit **(CA, 2003; MARDSR, 2016)** was fulfilled after 10 hours of yogurt fermentation.

Textural parameters like hardness, adhesiveness, cohesiveness, chewiness, gumminess, springiness and sensory parameters including taste, flavor and mouthfeel, appearance, and overall acceptance are important for consumers during the shelf-life of the yogurt **(Mousavi et al., 2019)**, this was a logical reason, why we determined textural properties of both yogurths. The results of textural analysis are presented in **Table 2**. We found statistically significant differences (p <0.05) in all textural parameters and total viable counts 24 hours after fermentation. The textural parameters have reached the following mean values (YoFlex YF-L812 and Delvo® Fresh YS-41): hardness 313 and 379 g, consistency 7725 and 9990 g.s^{-1}, cohesiveness -317 and -402 g, viscosity -633 and -788 g.s^{-1}. The higher the hardness value of the yogurt, the stronger the yogurt was. We found that yogurt with Delvo® Fresh YS-241 was harder, more consistent, cohesive and viscose in comparison with YoFlex YF-L812 yogurt at selected experiment conditions, which were proposed by the dairy company. The protein matrix had an important role in cohesiveness **(Tunick, 2000)** and can be affected by the starter culture used for yogurt fermentation.

The results of sensory testing are presented in figures 3 – 8. YoFlex YF-L812 is marked as sample A and Delvo® Fresh YS-41 is marked as sample B.

According to **Figure 3,** sample A had aroma and color very good, the taste good and the consistency was satisfactory. Sample B had taste, consistency, and color very good, the smell good. The difference in taste, consistency, and flavor between both samples. According to **Figure 4** both samples had the typical yogurt flavor and acid flavor. Sample A appeared less sweet. The sensory evaluation of consistency of yogurts is presented in **Figure 5**. We can see a difference only in the softness indicator. The sensory evaluation of overall appearance is presented in **Figure 6**. Both samples were evaluated as very similar, well clothed, soft look, and thick. The sensory evaluation of texture in the mouth is presented in **Figure 7**. Sample A was evaluated as less soft and both samples were not lumpy. The sensory evaluation of taste is presented in **Figure 8**. Both your didn't differ in all taste descriptors.

Even though appearance, texture, and thickness are very important characteristics to contribute to the quality of yogurt, the flavor is generally considered as the most important of all and critical indicators of consumer acceptability **(Olugbuyiro and Oseh, 2011)**. White yogurt with a high consumer acceptance should, in general, have a smooth, uniform, and spoonable texture, and be free from lumps, graininess, and visual whey separation **(Lucey and Singh, 1997; Lucey, 2004)**. Milk heated at pH 6.7 contains significant proportions of both bound and soluble denatured whey protein complexes and this treatment produced the stiffest yogurt gels **(Ozcan, Horne and Lucey, 2015)**.

Clean and typical yogurt flavor is very important. Acetaldehyde, diacetyl, and lactic acid are considered as the major aroma components of yogurt, but also other components, like acetone, acetoin, and acetic, formic, butanoic, and propanoic acids, have been listed as contributors to yogurt flavor **(Routray and Mishra, 2011)**. Yogurt starters that allow the development of acetaldehyde with restricted post-acidification and post-proteolytic activity are favorable **(Jørgensen et al., 2019)**.

Yogurt with YoFlex YF-L812 had better flavor. On the other hand, based on the sensory evaluation we can conclude that yogurt with Delvo® Fresh YS-241 culture had slightly better consistency and taste. We identified, that the flavoring of both yogurts was better after 24 hours of fermentation in comparison with continued fermentation time. This is in agreement with **Görner and Valík (2004)**. These authors found, that formation of flavorings, in particular acetaldehyde, is mainly related to *L. delbrueckii* ssp. *bulgaricus* and is also associated with acidification. It starts at pH 5.0, increases vigorously at pH 4.4 to 4.3 in 3 h at 42 °C, then slows down significantly and stabilizes at pH 4.0.

When the pH drops below pH 5, micelles of caseins, which are amphiphilic proteins, loses its tertiary structure due to the protonation of its amino acid residues. The denatured casein proteins reassemble by interacting with other casein proteins, and these intermolecular interactions result in a network of molecules that provides the semisolid texture of yogurt **(Zourari, Accolas and Desmazeaud, 1992)**.

We agree with these authors, because, it was clearly evident, that textural parameters are developed during the syneresis process and confirmed by analysis of texture. It is not possible to analyse the texture at first stages of syneresis with the disc-shaped probe with back extrusion. We made the texture analysis 24 hours after fermentation started.

Depending on the type of yogurt, the incubation process is done either in a large tank of several hundred liters or in the final individual plastic or glass containers. The textural parameters of stirred yogurt are different in comparison with set yogurt. Stirred yogurt is fermented in the bulk tank, mixed and then poured into the final selling containers.

Set yogurt, also known as French style, is allowed to ferment right in the container it is sold in.

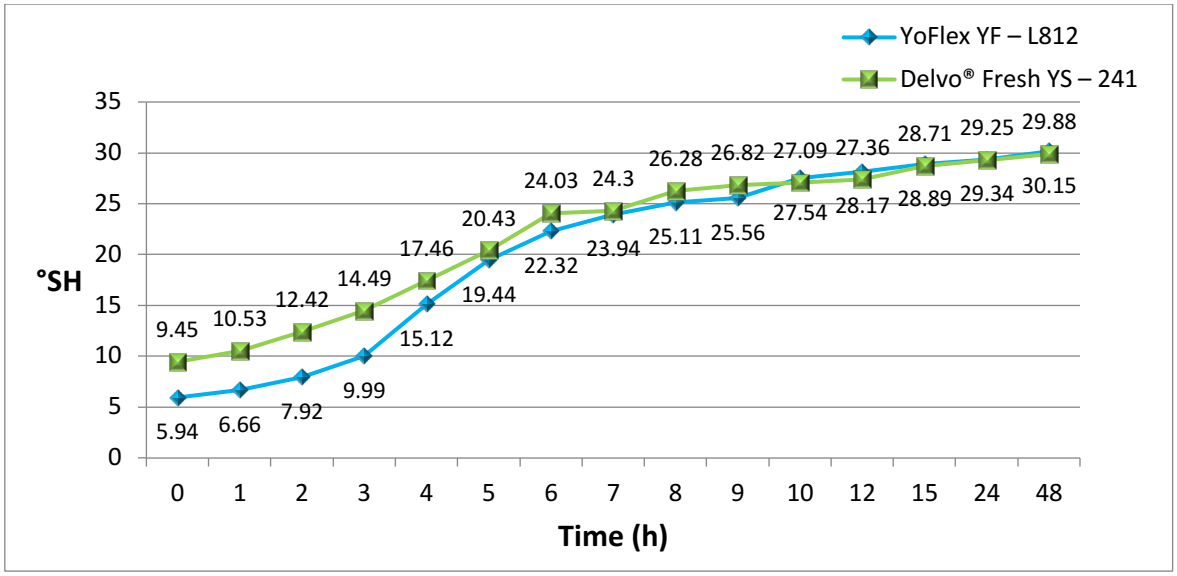

Figure 1 Development of titration acidity in white yogurts.

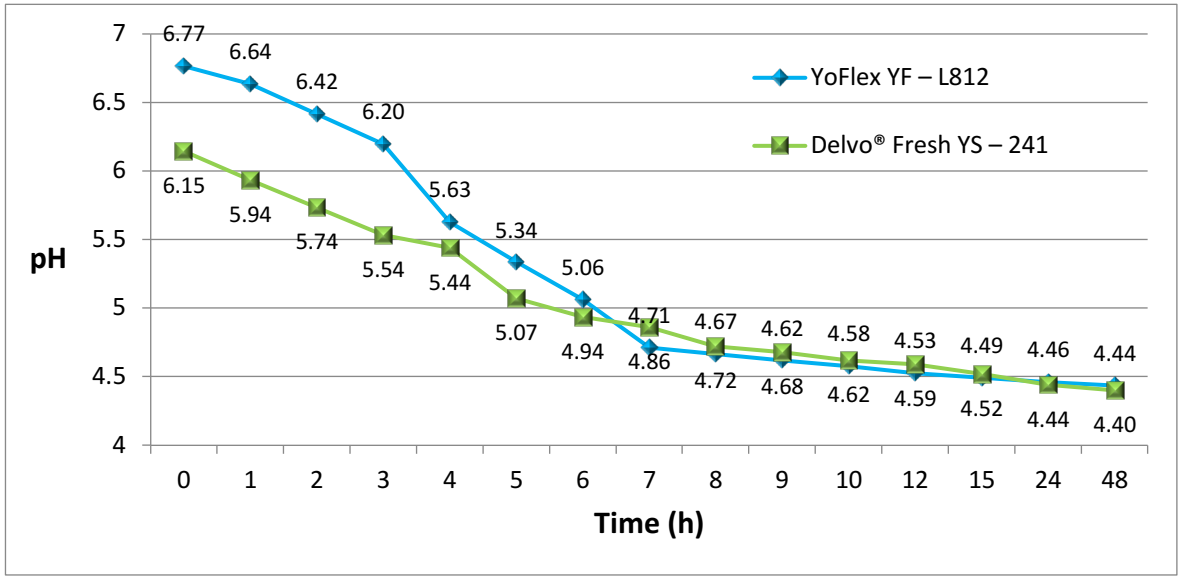

Figure 2 Development of pH in white yogurts.

Table 1 Total viable count in white yogurts (TVC.mL^{-1}).

Time (h)	Samples	
	Yogurt YoFlex® YF – L812	Yogurt Delvo® Fresh YS – 241
0 – milk with starter culter	2.16×10^2	2.18×10^2
5	3.42×10^5	2.37×10^5
10	4.81×10^7	5.21×10^7
24	6.28×10^7	7.14×10^7

Table 2 Texture properties of white yogurts.

Texture parameter	Yogurt with starter culture		
	YoFlex® YF – L812	Delvo® Fresh YS – 241	*p* value
Hardness (g)	313.118 (±38.452)	379.387 (±42.675)	0.05
Consistency (g.s^{-1})	7724.560 (±732.214)	9990.491 (±864.113)	0.05
Cohesiveness (g)	-317.405 (±39.326)	-402.404 (±49.783)	0.05
Viscosity (g.s^{-1})	-633.322 (±56.657)	-788.210 (±52.187)	0.05

Note: data in table represent the mean values and standard deviation.

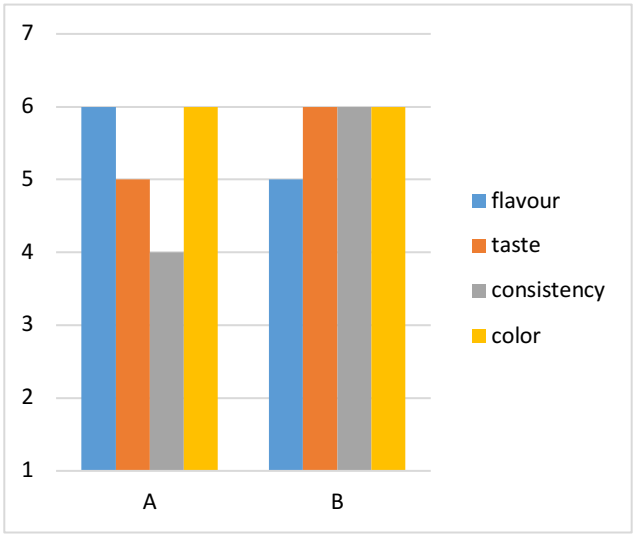

Figure 3 Sensory evaluation by quality indicator.

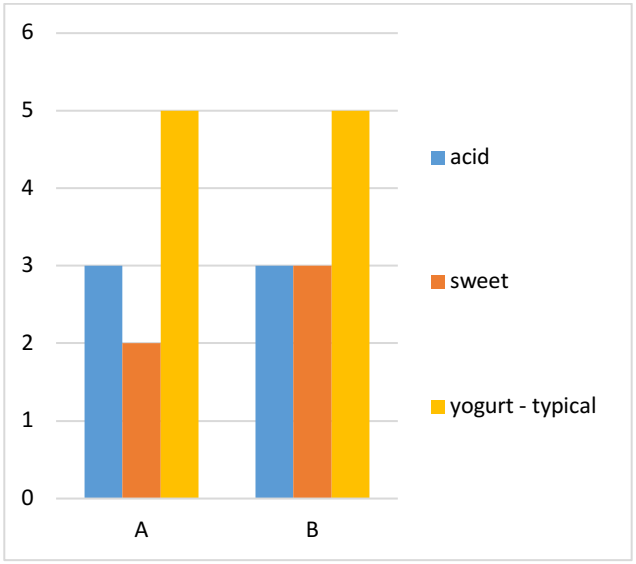

Figure 4 Sensory evaluation of flavour.

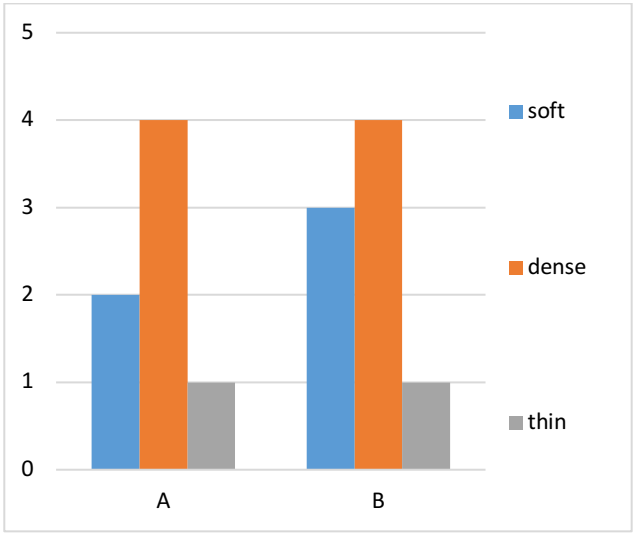

Figure 5 Sensory evaluation of consistency.

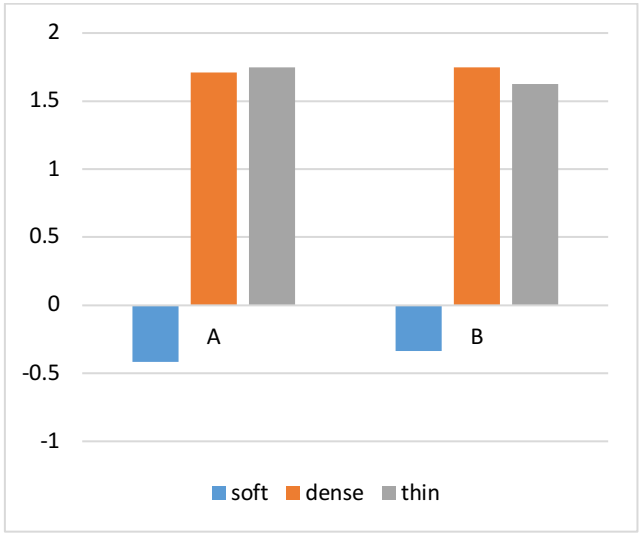

Figure 6 Sensory evaluation of overall appearance.

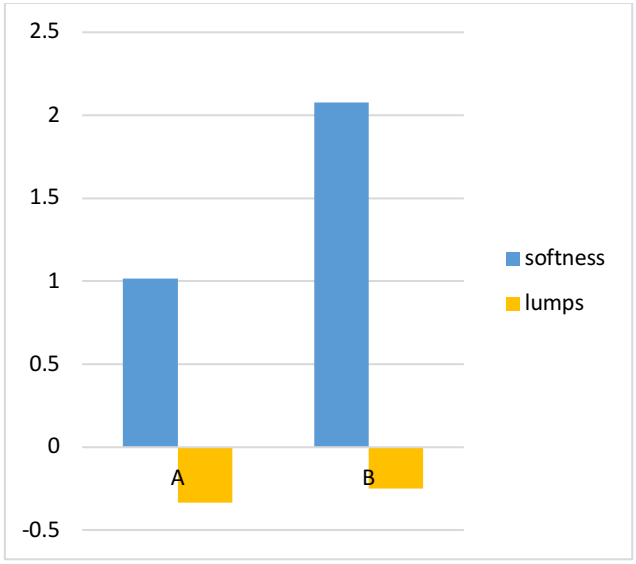

Figure 7 Sensory evaluation of texture in mounth.

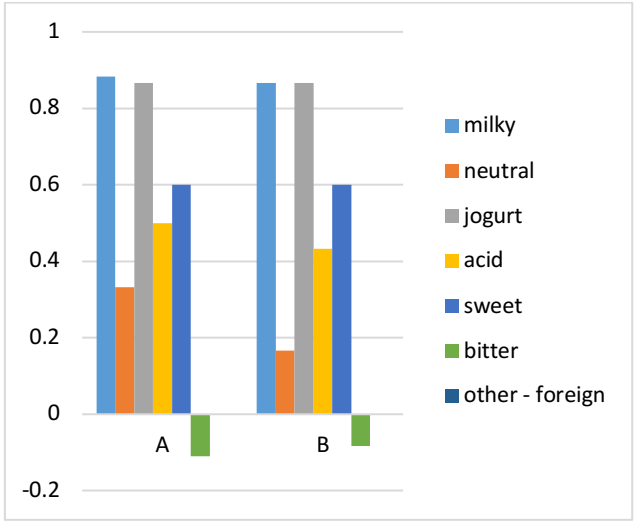

Figure 8 Sensory evaluation of taste.

In both instances, the lactic acid level is used to determine when the yogurt is ready for sale. The acid level is found by taking a sample of the product and titrating it with sodium hydroxide. A value of at least 0.9% acidity (29 °SH – 40 °SH) and a pH of about 4.4 are the current minimum standards for yogurt manufacture.

Industrial yogurt is produced by fermentation of pasteurized milk after inoculation with starter bacteria. These convert lactose to lactic acid. This changes the pH of the milk and coagulates at a certain pH. Industrial milk fermentation in yogurt production takes about 3 hours at 42 – 45 ºC. After this time, the yogurt must be cooled to a storage temperature below 10 ºC in order to interrupt the fermentation process. Durable yogurts are treated with pasteurization, which kills bacteria cultures and other microorganisms. Such treated yogurt loses biological value, mineral usability, and curative effects, especially antibacterial **(Tamime, Robinson, 1999)**.

When the yogurt reaches the desired acid level, it is cooled (10 – 12 °C), modified as necessary, and dispensed into containers (if applicable) **(Tamime and Robinson, 2000)**.

Manufacturing of yogurt has changed from crude and elementary procedures to more controlled procedures over time. A few of the advances in our knowledge that made this possible include use of ingredients in addition to milk, the use of starter cultures, and modern technologies **(Aryana and Olson, 2017)**.

CONCLUSION

Thermophilic starter cultures YoFlex® YF - L812 and Delvo® Fresh YS – 241 are suitable for yogurt production. The fermentation process using these starter cultures is slightly different. We have found, 4.4 was reached after 16 and respectively 15 hours of fermentation. We did not found statistically significant differences (p >0.05) in titration acidity of both yogurts after 7 hours of fermentation. We did not found statistically significant differences (p >0.05) in the pH of both yogurts after 7 hours of fermentation. We found statistically significant differences (p <0.05) in all textural parameters (hardness, consistency, cohesion, and viscosity). The textural parameters have reached the following values: hardness 313 and 379 g, consistency 7725 and 9990 $g.s^{-1}$, cohesion -317 and -402 g, viscosity -633 and -788 $g.s^{-1}$. The total viable count of microorganisms in yogurts after 24 hours of fermentation was 6.28×10^7 $KTJ.g^{-1}$ and 7.14 $KTJ.g^{-1}$ respectively. Based on the sensory evaluation we can conclude that yogurt with Delvo® Fresh YS-241 culture had slightly better characteristics than yogurt with YoFlex YF-L812.

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BIOCHEMICAL COMPOSITION OF THE HOPS AND QUALITY OF THE FINISHED BEER

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ABSTRACT

The large varieties of hops and hop products used in the brewing industry. Various in the biochemical composition, individual approaches to the brewing technology of each hop product are required in order to obtain a high-quality beer with a characteristic bitter taste and aroma. The purpose of this work was to study the biochemical composition of pressed conical hops, pellets of hop type 90, type 45, ethanolic and CO_2 extracts of hop of various varieties, and their influence on the quality of the finished beer.

As a result of comprehensive research on hops and hop products of various varieties, using the modern biochemical methods were determined differences in their biochemical composition depend on the absolute values such parameters as the mass fraction of α-acids, β-acids and their composition, xanthohumol, general polyphenols, essential oils, the ratio of their valuable components of hops: β-acids to α-acids and also for quantity of general polyphenols, essential oils per unit of α-acids. Based on the results of the biochemical composition of hops and hop products were investigated their influence on the quality of beer and were determined their using in brewing.

Keywords: Hops, Products, Beer, Biochemical, Composition

INTRODUCTION

One of the main and irreplaceable type of raw material for brewing is hop. Its constituent substances gives beer a specific taste and aroma, promote foam formation and stability quality of beverage. The quality of hop and hop products do not depend on only the quality of beer, but also on the efficiency of brewing production in general. The quality of hop do not directly relate only with varietal characteristics and conditions of cultivation, but also with conditions of post-harvest handling and storage **(Lyashenko, 2002; Pavlovič et al., 2012)**.

High-quality beer with characteristic bitter taste and aroma obtains only with the use of hops and hop products with a certain biochemical composition. At the same time, the efficiency of extraction, isomerization and transformation of separate numerous hop compounds in the process of boiling beer wort is very important **(Protsenko et al., 2012)**.

The using of hop and their products in the brewing is due to the fact that its cones contain large quantity of biologically active substances such as bitter substances, polyphenols and essential oils. Specific substances give to beer a unique bitterness and a specific aroma, participate in the clarification and formation of foam, increase its stability during storage **(Protsenko et al., 2018)**.

The beer production in Ukraine and in the world increasing that stimulates the demand for hop products and necessitates the balanced development of the industry for satisfaction of the brewing industry. However, in a competitive struggle on the market, the winner is not the one who produces more but average hops, but the one who makes it better in quality and cheaper in the price. Even under the current difficult conditions, there are opportunities and reserves for the production of high-quality commodity hops, including aromatic **(Bober et al., 2015)**.

For brewing used about 90 % of hop and hop products. The demand for hop is constantly increasing, but the industry is far from satisfying the domestic market. Currently, the Ukrainian brewing industry used up to 20 % of domestic production. The main part of raw materials is imported, which create dependence on world market conditions. This industry divide inter countries such as Germany, Czech Republic, Poland, Austria, USA, Canada, Australia and China **(Pryimachuk et al., 2018)**.

As marketing research has shown to the world market delivered only about 10 % of natural cones hops but 90 % of granulated and hop processed into extracts from total amount of obtained hop products. Only in the brewery of low-power remained classical beer production technology,

where for brewing beer wort traditionally used pressed hops. Powerful breweries of Ukraine transferred to using different types of pellets, ethanolic and CO_2-extracts of hops **(Zheplinska et al., 2019)**.

The most rational way of processing hop cones is to granulate them. Only this technology provides for a more complete save of all complex of valuable substance hops for a long time before application in the production of beer, more stable use, high quality of the finished product. The most popular and promising are pellets type 90, which according to the chemical composition practically do not differ from the native cones of hops.

Pellets type 45 apply to enriched hop products. They differ significantly in the biochemical composition from native hops, since the amount of polyphenols per unit of alpha acids is halved, and their production associated with additional losses of essential oils. For brewing properties, it is intermediate product between cones and CO_2-extract. Granulated hops are also more convenient to use for packing and adding to wort. In addition, the volumetric mass of granulated hops is much smaller than compressed, thus reducing transport and storage costs **(Zheplinska et al., 2019)**. Another way that make it possible to keep bitter and other valuable substance hops in the unchanged state, it is production of hops extracts with using organic and inorganic solvents. The expediency of producing hop extracts is conditioned by the possibility of obtaining high-quality and efficient products that can be stored for a long time without changing their biochemical composition **(Protsenko and Litvynchuk, 2017)**.

However, despite the fact that in the world more than 90 % of native (cone) hops processed into hop products, there is absent of scientific research about quality hop varieties which have different content and composition of bitter substances, polyphenols and essential oils. The specialists in agriculture and the brewing industry need to know the main advantages and disadvantages of these products and their impact on the quality of the finished beer, especially as in the literature, especially in advertising, as a rule, they write more about their advantages, without emphasizing the disadvantages.

The purpose of this work was to study the biochemical composition of hops and hop products during 2012 – 2017 of various hop varieties and their impact on the quality of the finished beer.

Scientific hypothesis

Studies of hops and hop products of different breeding varieties with using modern biochemical methods will supply to establish differences in their biochemical composition. Peculiarities of the biochemical composition of hop products will allow individually to approach the brewing technology of each hop product in order to obtain high quality beer with a characteristic bitter taste and aroma.

MATERIAL AND METHODOLOGY

Samples

For researches, cones of pressed hops and pellets of type 90 typical representatives of the aromatic group of varieties (Slavyanka, Nationalny, Zagrava) and bitter (Alta, Hercules) which widespread in the production conditions of Ukraine, were used; pellets type 45 varieties Tradition and Spalt Select; ethanol and CO_2-extracts of the Hercules variety of foreign production.

Beer from the samples of hop products was manufactured at the mini brewing biochemistry department of hop and beer of the Polissya Institute of Agriculture of the National Academy of Agrarian Sciences of Ukraine with the output of 100 litres, which adequately reflects the conditions of real breweries.

The hop and hop products added on the mini brewery added on the content of α-acids in them.

The wort was prepared from 100 % barley malt. The norm of hop was calculated in the amount of 50 mg of bitter substances per 1 dm^3 of wort. Duration of wort boiling with hop products was 90 minutes.

Methods

Modern international physical-chemical methods of determining the quality indices of hops and hop products and products of their transformation in the brewing process were used: high-performance liquid chromatography, spectrophotometry and also methods of quality control of beer wort and finished beer, harmonized with the methods of the European Brewery Convention **(Jaskula, et al., 2007)**.

The content and composition of α- and β-acids and xanthohumol were determined by high-performance liquid chromatography. Chromatography was performed using Ultimate 3000 liquid chromatograph with a UV detector at a temperature of 35 °C of the method described in state standard of Ukraine 14164:2019 **(DSTU EN 14164:2019)**. The 100 × 0.0021 m column was used which was filled with a Pinacle sorbent DV E18 3 μ. As a mobile phase, a solution of methanol, water and acetonitrile was used in the ratio of 38:24:38. For the quantitative determination of xanthohumol was used standard-ethanol of xanthohumol with containing 99.8 % of this compound and for α- and β-acids used the international standard franchise (ISF-3) **(Jaskula, et al., 2007)**.

The amount of essential oil was determined by the Ginsberg method **(Lyashenko, 2002)**. The qualitative composition of the essential oil was determined by the gas-liquid chromatography method on 50-60 m capillary quartz columns of chromatograph "Crystal 2000 M". The total amount of polyphenolic compounds and proanthocyanidins was determined by photometric methods in the modification of M.I. Lyashenko **(Lyashenko, 2002)**.

The index of oxidation of bitter substances in hops and hop products was determined by extraction of bitter substances with the following definition on spectrophotometer in an extract of an optical density of oxidized components at wavelength was 275 nm, and a- and β-acids at a wavelength was 325 nm **(Lyashenko, 2002)**.

In beer wort and beer, bitterness was determined by spectrophotometric method of EBC. The method is based on the determination of the optical density of isooctane obtained by removing bitter substances from acidified wort or beer from isooctane (2,2,4-trimethylpentane), with used wavelength is 275 nm. The magnitude of the bitterness expressed by units of the international scale with determining bitterness according to EBC which was

evaluated on the based the index of optical density. The content of polyphenolic compounds in the wort and beer, were measured on a spectrophotometer according to EBC 8.11 and EBC 9.11. **(Kábelová-Ficová et al., 2017)**.

The quality of beer was evaluated organoleptically by tasting of tasting commission according to the requirements for beer according to the branch Instruction by the 25-point integrated estimation **(Punčochářová et al., 2019)**.

Statistic analysis

The mathematical processing of the data was performed with using the method of dispersion (ANOVA) analysis using the programs Statistica 10 and Microsoft Office 2010.

RESULTS AND DISCUSSION

Complex biochemical studies of hops and hop products of various varieties with the use of modern biochemical methods (high performance liquid chromatography) made it possible to establish that hops and hop products of different varieties have different biochemical composition and hence a different brewing value.

The biochemical characteristic of the widespread hop products in the brewing industry was presence in Table 1.

The most valuable compounds of hops and their processing products are bitter substances. Bitter substances are unique and do not presence in the other plants. The most important among the bitter substances are alpha acids, which converted into iso-alpha acids, the main compounds of bitterness of beer, during the process of isomerization at boiling of beer wort.

When added to beer of fresh hops, almost 90 % of bitterness of beer is formed as a result of isomerisation of alpha acids in iso-alpha acids **(Malowicki and Shellhammer, 2006)**. The quantity of alpha acids is the main pricing factor for the evaluation of hops and hop products. The content of α-acids in the studied hop products changed from 3.82 to 52.83 % (Table 1). The highest content of α-acids was found in the CO_2-extract of the Hercules variety – 52.83 %. Among pellets, this index was the maximum in pellets of hop type 90 bitter grade Hercules – 13,64 %.

Beta-acids are not bitter in taste, but in the process of oxidation compounds are formed having a pleasant bitterness. One of their main properties is a high antiseptic effect, which important for increasing the biological stability of beer during storage. Therefore the beta-fraction of hops abroad used in the processing of sugar beets to improve their storage in sugar factories **(Lyashenko, 2002)**. Depending on the variety and type of hops, the content of β-acids ranges from 3.41 till 18.81 %.

As the researches shown, hop pellets of type 90 of domestic production contain the whole complex of necessary for brewing substances and equivalent to hop cones. Characteristic feature of cones hop and pellets of type 90 and type 45, in particular of aromatic varieties, is the high positive coefficient of aromatics between the content of β- and α-acids which ranged from 0.89 till 1.79. This is a main feature in the estimation of the brewing quality of hops and hop products. On average, conducting research in 2012 – 2017 the higher indicator of aromatics were characterized as cones and hop pellets of the Slavyanka and Nationalny varieties. Unlike, the cone-shaped and granulated hop of aromatic varieties, cones and pellets of hops of bitter varieties, characterized by a sharp aroma and high content of α-acids. The ratio of β-acids to α-acids in the cones and pellets of hop of bitter varieties and extracts was less than 1. From 0.26 in hop pellets type 90 of the Hercules variety to 0.53 in hop pellets type 90 of the Alta variety.

Application of high-performance liquid chromatography in our studies allow to established the quantitative and qualitative composition of bitters substances of hops and hop products of various grades (Figure 1, Figure 2 and Figure 3). On the presented chromatograms the composition of bitters substances of the studied hop products clearly shows the difference in their quantitative and qualitative composition. The chromatograms of the composition of bitters substances of investigated hop products, we can see difference between quantitative content of a- and β-acids, and in their composition. The composition of the α- and β-acids, depending on the type of hop cones and the type of hops, changed significantly, and the first it related with the content of cohumulon in the α-acids and the colupulon in β-acids. Thus, the mass fraction of cohumulon in the a-acid content ranged from 22.72 till 38.62 %, and the colupulon - from 42.61 till 59.22 %. As a rule, if more contained in the a-acid cohumulon, than the higher the content of the colupulon was in the β-acids.

The composition of a- and β-acids is important for the production of high-quality beer. The most quality for brewing has hops with mass fraction of cohumulon in the composition of a-acids does not exceed 30 %. Hops with content cohumulon of 40 – 50 % in alpha acids used mainly for the preparation of isomerized preparations, because it is not used in the natural form for the production of beer **(Malowicki and Shellhammer, 2006)**.

Brewers pay particular attention the index of oxidation of bitters substances, because they consider it one of the main indicators of the quality of cones of hops and hop products. This index on a par with the content of alpha acids controlled during the purchase of consignments of hop and hop products. Than the lower index of oxidation of bitters substances then the higher were quality of hop products **(Michałowska, 2017)**.

According to Table 1, the index of oxidation during the years of investigation, both in cones, pellets hops, and hop extracts, fluctuated within 0.27 – 0.50. The minimum index of oxidation is set in the CO_2 extract – 0.27 but the maximum in the pellets type 90 of hop Slavyanka variety - 0.50 and did not exceed the norms of normative documents. It was established that the index of oxidation in the hop pellets was not higher than it was in the cones of hop. This indicates that the quality of the pellets of the hops type 90 is practically the same as the cones hops but the quantitative and qualitative composition of the bitter substances depends on the variety from which they obtained.

The data which we received about the content of xanthohum in the biochemical composition of hops and hop products of various varieties deserved attention (Table 1, Figure 1, Figure 2 and Figure 3).

Table 1 Biochemical characteristics of hops and hop products for brewing.

Samples of hops and hop products	a-acids, %	Kohumulon within α-acids,%	β-acids, %	Colupulon within β-acids, %	Ratio of α/β – acids (method EBC 7.7)
Cones of hops of the Slavyanka variety	3.82 ±0.04a*	22.72 ±1.16 a	5.42 ±0.07 a	42.63 ±0.86 a	1.79 ±0.02 a
Cones of hops of the Nationalny variety	5.40 ±0.08 b	25.31 ±0.98 ab	5.43 ±0.09 a	44.30 ±1.10 ab	1.15 ±0.08 b
Cones of hops of the Zagrava variety	5.71 ±0.14 c	24.03 ±1.23 ab	4.72 ±0.05 b	42.61 ±0.65 a	1.06 ±0.05 c
Cones of hops of the Alta variety	8.83 ±0.06 d	24.52 ±0.80 ab	4.03 ±0.02 c	44.82 ±0.72 ab	0.52 ±0.01 d
Pellets of type 90 Slavyanka variety	4.64 ±0.12 e	25.21 ±0.76 ab	4.22 ±0.06 ec	43.42 ±0.88 ab	1.28 ±0.04 e
Pellets of type 90 Nationalny variety	6.30 ±0.09 fi	25.62 ±1.08 b	5.13 ±0.10 d	43.54 ±1.18 ab	1.17 ±0.05 b
Pellets of type 90Zagrava variety	6.41 ±0.10 f	24.32 ±0.68 ab	4.32 ±0.08 ec	44.80 ±1.22 ab	0.92 ±0.08 f
Pellets of type 90 Alta variety	10.1 ±0.08 g	28.31 ±1.34 c	4.33 ±0.04 ec	46.22 ±0.78 b	0.53 ±0.02 g
Pellets of type 45 Tradition variety	8.20 ±0.04 h	26.22 ±1.12 bc	6.32 ±0.08 f	45.32 ±0.65 ab	0.92 ±0.10 f
Pellets of type 45 Spalt Select variety	6.23 ±0.13 fi	24.81 ±1.10 ab	4.32 ±0.05 ec	43.34 ±1.08 ab	0.89 ±0.11 f
Pellets of type 90 Hercules variety	13.64 ±0.12 j	33.33 ±1.62 d	3.41 ±0.04 g	54.04 ±1.36 c	0.26 ±0.07 h
Ethanol extract hops of the Hercules variety	41.82 ±0.10 k	38.50 ±1.74 e	17.02 ±1.11 h	59.22 ±1.20 d	0.38 ±0.05 i
CO_2-extract hops of the Hercules variety	52.83 ±0.07 l	38.62 ±1.42 e	18.81 ±1.10 i	59.13 ±1.05 d	0.34 ±0.04 i
minimum	3.82	22.72	4.03	42.61	0.34
maximum	52.83	38.62	18.81	59.22	1.79
mean	13.38	27.81	6.73	47.18	0.86

Samples of hops and hop products	Oxidation index, I_o	General polyphenols, %	Essential oil, mg/100 g	Xanthohumol, %	-
Cones of hops of the Slavyanka variety	0.34 ±0.01 a	5.02 ±0.12 a	0.78 ±0.05 a	0.35 ±0.02 a	-
Cones of hops of the Nationalny variety	0.37 ±0.01 b	5.53 ±0.08 b	0.48 ±0.05 b	0.49 ±0.01 b	-
Cones of hops of the Zagrava variety	0.33 ±0.01 a	6.62 ±0.14 c	0.67 ±0.02 c	0.35 ±0.01 a	-
Cones of hops of the Alta variety	0.36 ±0.00 ab	5.94 ±0.10 d	1.22 ±0.04 d	0.25 ±0.03 c	-
Pellets of type 90 Slavyanka variety	0.50 ±0.02 c	6.03 ±0.05 d	0.36 ±0.07 e	0.40 ±0.02 d	-
Pellets of type 90 Nationalny variety	0.41 ±0.03 d	4.92 ±0.18 a	0.51 ±0.08 b	0.49 ±0.04 b	-
Pellets of type 90Zagrava variety	0.45 ±0.01 e	6.54 ±0.06 c	0.49 ±0.02 b	0.40 ±0.02 d	-
Pellets of type 90 Alta variety	0.40 ±0.02 d	4.52 ±0.05 e	0.87 ±0.04 f	0.35 ±0.02 a	-
Pellets of type 45 Tradition variety	0.47 ±0.01 e	11.03 ±0.07 f	0.22 ±0.05 g	0.52 ±0.01 b	-
Pellets of type 45 Spalt Select variety	0.49 ±0.02 ce	9.92 ±0.18 g	0.33 ±0.03 h	0.45 ±0.03 b	-
Pellets of type 90 Hercules variety	0.37 ±0.03 b	4.21 ±0.04 e	0.52 ±0.02 b	0.45 ±0.02 b	-
Ethanol extract hops of the Hercules variety	0.40 ±0.01 d	-	0.75 ±0.01 a	2.10 ±0.05 e	-
CO_2-extract hops of the Hercules variety	0.27 ±0.02 f	-	2.5 ±0.06 i	-	-
minimum	0.27	4.21	0.22	0.25	-
maximum	0.50	11.03	2.50	2.10	-
mean	0.40	6.40	0.75	0.55	-

Note: *value ±SD are means of three repetitions, *Mean values followed by different letters are statistically different at $p < 0.05$.

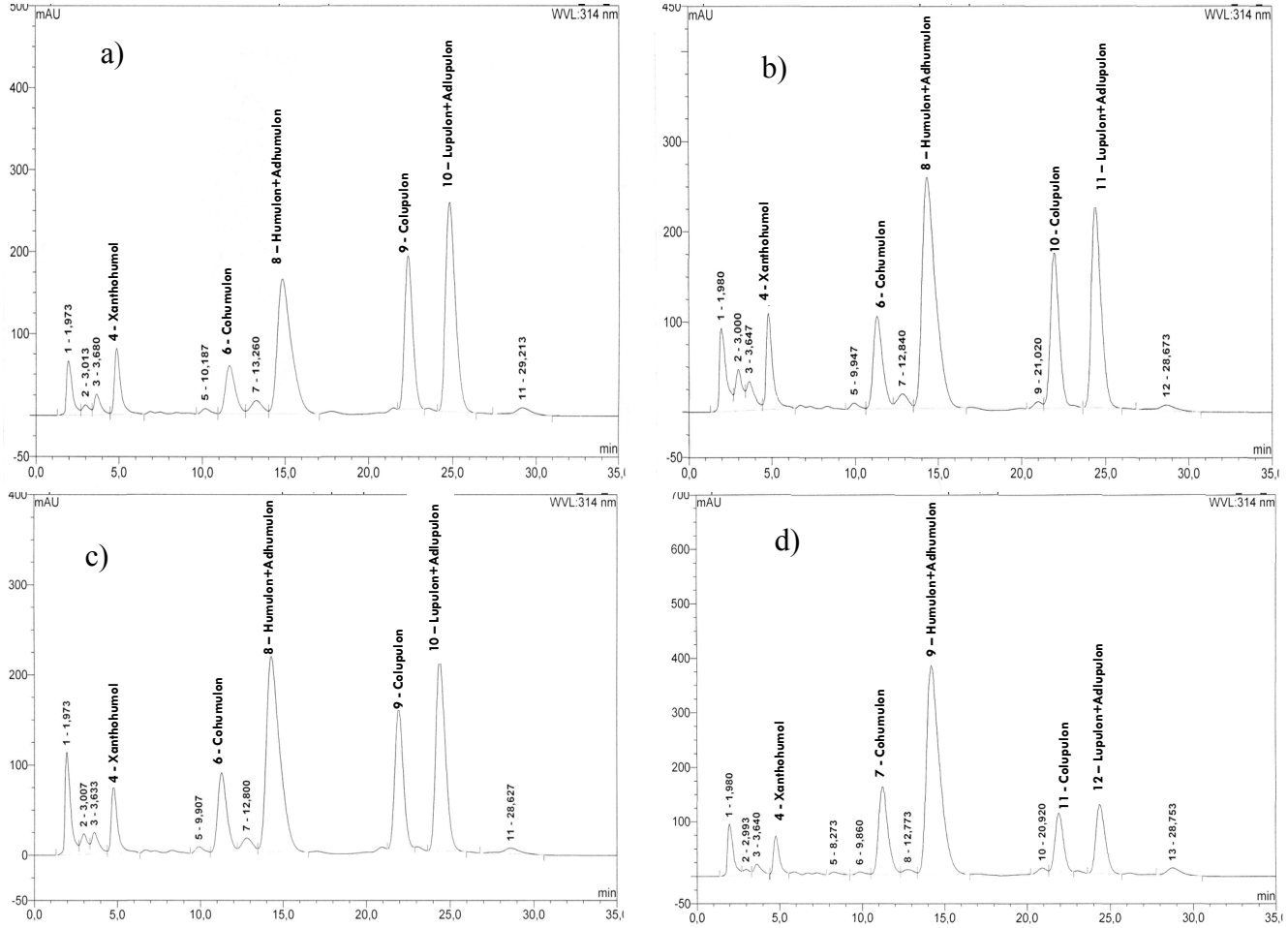

Figure 1 Chromatograms of the composition of bitters substances of cones of hops of various varieties: **a)** Slavyanka variety; **b)** Nationalny variety; **c)** Zagrava variety; **d)** Alta variet.

Depend on the variety of hops and the type of hops products, the amount of xanthohumol ranged from 0.25 till 2.10 %.

The highest content of xanthohumol was found in the ethanol extract of Hercules (2.10 %) and pellets of hop type 45 of the Tradition variety (0.52 %). The quantity of xanthohum in hops and hop products independent from content of bitter substances in the α-acids and β-acids were established.

Thus, in the cones and pellets of the type 90 of the bitter Alta variety with a high content of α-acids, the content of xanthohumol was smallest, which indicate on the absence correlation between the content of α-acids and xanthohumol.

As we seen from the chromatogram (Figure 3), in the biochemical composition of the CO_2 extract of variety Hercules xantohumol was absence.

According to literary sources **(Lyashenko, 2009)**, at the production of ethanol extract it contains at least 90 % of prenylflavonoids (including xanthohumol) of hop but at the production of CO_2-extract, these substances are practically not extracted and remain in the wastes.

Nowadays in the world there are intensive scientific researches about medicinal properties of this compound.

The experimental data obtained concerning the therapeutic effect of xanthohumol indicate that it was quite effective in the treatment of fungi, staphylococci, streptococcus, herpes and hepatitis viruses and has anti-cancer properties. Xanthogumol has negative affect on cancer cells of a person with a diagnosis of large intestine, breast, ovaries, prostate and leukemia, and does not affect on the healthy cells, activates enzymes that inhibit tumor growth, neutralizes the effect of enzymes that contribute to their growth, inhibit the growth of metastases **(Ferk, et al., 2010; Yang et al., 2013)**. The anti-carcinogenic effect of xanthohumol associated with its high antioxidant properties. Such an assessment of the effect of xanthohumulin was confirmed in the German Cancer Center **(Shanina et al., 2019)**.

In the process of beer preparing xanthohumol converted into isoxantohumol, which also has anti-carcinogenic properties **(Żołnierczyk, et al., 2017)**. However, the anti-carcinogenic action of isoxantohumol is 10 % less than xanthohumol. At wort preparation about 70 % of xanthohumol converted to isoxantohumol and in the finished beer left only 30 %.

Therefore, brewers need to pay attention to this when developing technologies for making beer and soft drinks with high content of xanthohumol and isoxantohumol for the prevention of cancer. With aim increasing xanthohumol in the beer have to add to beer at wort process hop products with high content of xanthohumol and also it added at the end of beer boiling for prevent the process of isomerization of this component.

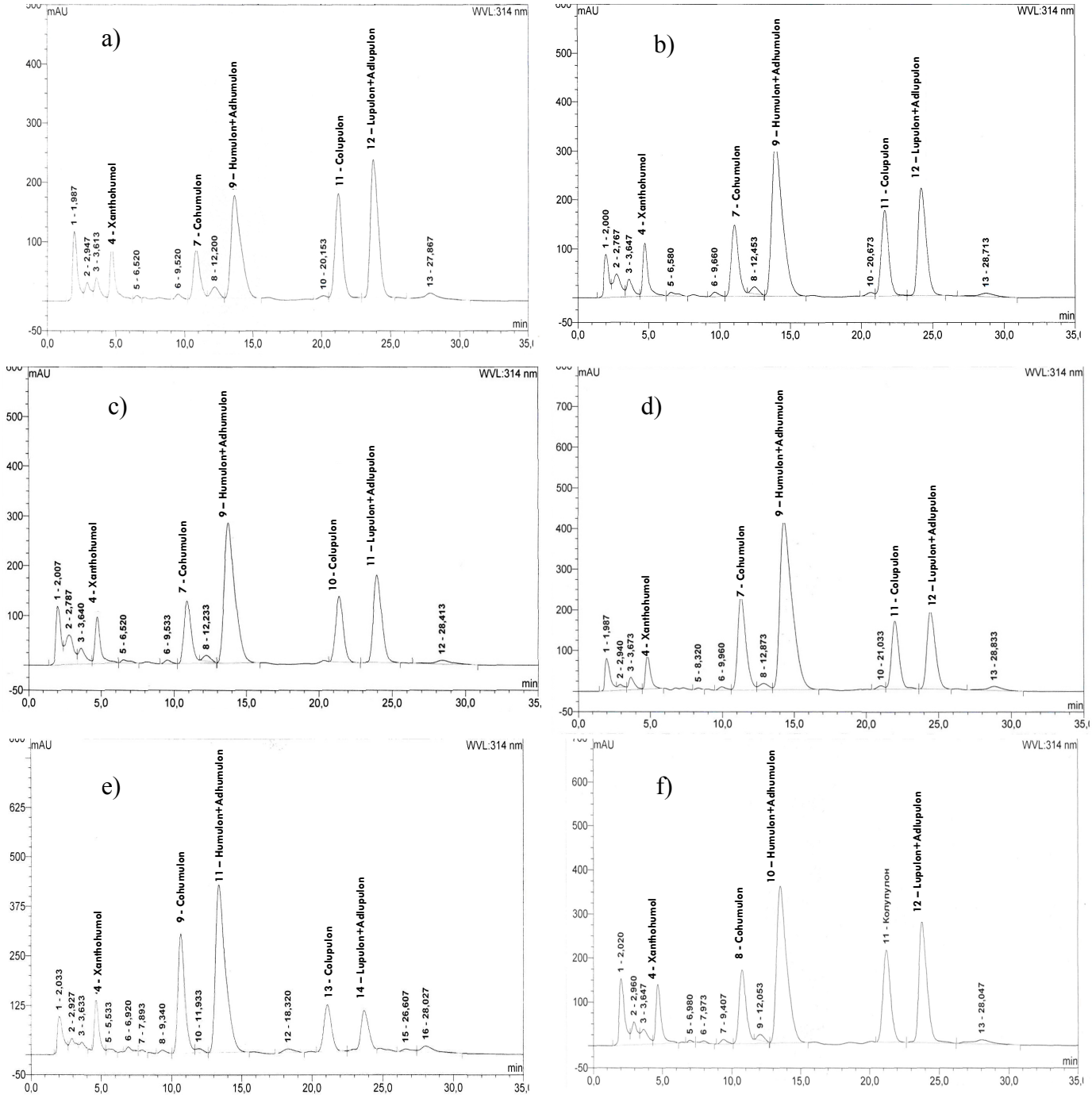

Figure 2 Chromatograms of the composition of bitters substances of pellets of hops: **a)** Pellets of type 90 Slavyanka variety; **b)** Pellets of type 90 Nationalny variety; **c)** Pellets of type 90 Zagrava variety; **d)** Pellets of type 90 Alta variety; **e)** Pellets of type 90 Hercules variety; **f)** Pellets of type 45 Tradition variety.

According to the results of the conducted researches (Table 1), the content of essential oils in the hop products was from 0.22 till 2.5 mg.100 g^{-1}. The highest content of essential oils was found in the CO_2 extract of hop of the Hercules variety. However, in brewing, in addition to the content and qualitative composition of essential oils have quantity of essential oil per 1 g of α-acids since the quantity of hops applied to beer calculated taking into account the content of α-acids.

On the contrary pellets and extracts, in cones of hops it is possible to see more quantity of essential oil per 1 g of α-acids, which provides more aromatic beer. For obtain the beer with a good hop flavour, it is necessary to use hops with content of essential oil at least 0.2 mL or about 50 μL sesquiterpenes per 1 g of α-acids **(Lyashenko, 2002; Pavlovič et al., 2012; Michałowska, 2017)**.

The organoleptic properties of beer made from different hop products also affect the different content of polyphenolic compounds. Along with the bitter substances, polyphenols have an important role in the forming of completeness and purity of the taste of the beverage, and also directly affect the freshness and stability of beer during storage. Hop polyphenols interact with high molecular proteins of wort, form complexes that fall into the precipitate and thus improve the clarification of wort and beer **(Goiris et al., 2014)**.

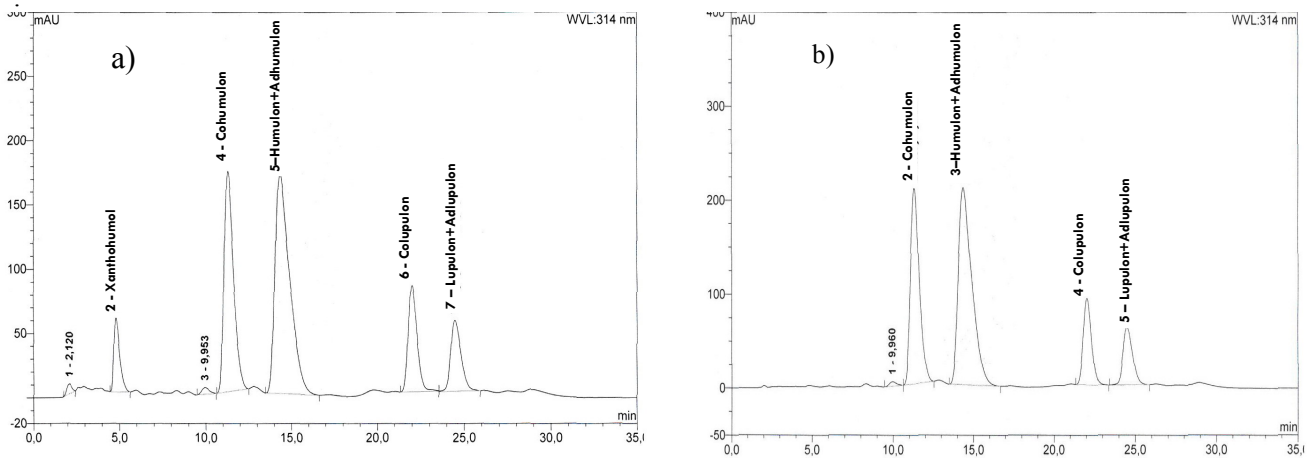

Figure 3 Chromatograms of the composition of bitter substances extracts of hops: **a)** Ethanol extract hops of the Hercules variety; **b)** CO_2-extract hops of the Hercules variety.

Always the best quality has beer which made from hops with a content of polyphenols do not less than 4.5 % **(Goiris et al., 2014)**.

The content of general polyphenols in the hop products according to Table 1 changed from 4.21 till 11.03 %. However, the main property of the hop products for this indicator determined do not by their general content, but by common quantity polyphenols per 1 g of α-acids. If in the cones of hop of aromatic varieties this value ranges from 1.0 till 1.3, hop pellets type 90 from 0.8 till 1.3, but in hop pellets type 90 bitter varieties from 0.3 till 0.4, that less on the 2.5 – 3.0 times. The studies showed that polyphenolic compounds were absent in ethanol and CO_2 extracts. Our studies confirmed investigations other scientists, which indicate the absence or insignificant content of polyphenolic compounds in these hops products.

In connection with this, for normal implementation of the brewing process and obtaining a full-fledged beer, it is necessary to add a certain quantity of cones or pellets of hop. Complex biochemical researches of hops and hop product of diffrent varieties with the used of modern biochemical methods give possible to established that hops and hop products of different varieties have different biochemical composition and hence different brewing value. This testified the fact that at the norms of different hop products of individual varieties with the same content of a-acids in the wort added different quantity of valuable for brewing components of hops. Consequently, the quality of beer produced by the same technology, but with the use of hops and hop products of different varieties can differ significantly.

Therefore, along with studies on the biochemical composition of pressed cones of hop and hop products of different varieties of domestic and foreign production, studies of their influence on the quality of the finished beer were conducted.

As the results of the researches, all obtained samples of beer were produced according the classical technology of light unfiltered beer which requirements of method described in state standard of Ukraine 3888:2015 **(DSTU 3888:2015)**. The physical and chemical indexes of the beer samples from the researches varieties of pressed cones of hop and different hop products presented in Table 2.

The analysis of physical and chemical indicators of beer quality shows that the degree of using of complex of valuable substances of hops and hop products was significantly higher in pellets and extracts than in native hop cones (Table 2). From the data which were presented we can see if used hop pellets type 90 of the Ukrainian bitter Alta variety in the hopped wort and beer, there were higher general indicators of bitterness and polyphenols than we using hops pellets type 90 of bitter Hercules type of German production. The norm at the same time was the same, taking into account only the content of alpha acids. Consequently, such results were due to the higher content of the beta fraction and polyphenols in pellets of the bitter Alta variety. The maximum amount of bitterness of beer provided pellets of hop type 45 of the Tradition variety, and the maximum amount of polyphenolic compounds in the wort and beer obtained with using hops cones and pellets of type 90 Slavianka and Zagrava varieties. Since there are not polyphenols in the extracts therefore in the wort and the finished beer which were produced from them obtained the least quantity of polyphenolic compounds.

In the process of basic and additional fermentation of beer the amount of bitter substances and polyphenols decreased **(Lyashenko, 2002; Pavlovič et al., 2012; Michałowska, 2017)**. Thus, the amount of bitterness from wort to finished beer decreased in the range of 14.5 – 23.3 % and total polyphenols 15.2 – 20.9 %.

The organoleptic assessment of the quality of the beer samples which were determined by the tasting commission, showed that was significantly different in taste, in the character of bitterness and aroma (Table 3).

All samples of beer (Table 3) were good or excellent quality and did not significantly differ. Each sample of beer had different taste, aroma or quality and fullness of bitterness. The quantity of pressed cones of hop (hop products) for adding to the wort calculated depend on the content of alpha acids in accordance with the branch Instruction TI 10-04-06-136-87 **(TI, 1998)**.

Table 2 Physico-chemical indexes of the beer samples.

No.	Grade of beer Variant of the experiment	Bitterness of wort, unit EBC	Content polyphenols in the wort, mg/dm^3	Bitterness of beer, unit EBC	Content polyphenols in the beer, mg/dm^3
1	**Cones of hop of the Slavyanka variety**	27.72 ±0.06 a*	232.42 ±0.22 a	21.60 ±0.64 a	202.10 ±0.15 a
	Cones of hop of the Nationalny variety	29.30 ±1.02 b	240.64 ±0.34 b	25.52 ±0.24 b	200.44 ±0.25 b
3	**Cones of hop of the Zagrava variety**	28.04 ±0.05 a	281.61 ±0.50 c	26.64 ±0.34 c	222.63 ±0.30 c
4	**Cones of hop of the Alta variety**	27.33 ±1.06 a	232.43 ±0.16 a	23.43 ±0.38 d	188.14 ±0.10 d
5	**Pellets of type 90 Slavyanka variety**	36.00 ±0.05 c	240.60 ±0.35 b	26.22 ±0.18 bc	202.92 ±0.15 a
6	**Pellets of type 90 Nationalny variety**	32.72 ±1.10 d	219.42 ±0.40 d	27.73 ±0.75 e	191.63 ±0.20 e
7	**Pellets of type 90 Zagrava variety**	29.44 ±0.04 b	244.30 ±0.18 e	27.32 ±0.24 ce	211.52 ±0.40 f
8	**Pellets of type 90 Alta variety**	29.90 ±0.05 b	188.92 ±0.26 f	26.04 ±0.35 bc	170.11 ±0.20 g
9	**Pellets of type 45 Tradition variety**	33.92 ±0.08 e	227.10 ±0.30 g	31.04 ±0.25 f	191.10 ±0.15 e
10	**Pellets of type 45 Spalt Select variety**	29.84 ±0.01 b	206.62 ±0.28 h	28.42 ±0.37 eg	194.33 ±0.10 h
11	**Pellets of type 90 Hercules variety**	33.20 ±0.06 de	191.93 ±0.17 i	28.64 ±0.40 eg	153.32 ±0.28 i
12	**Ethanol extract hops of the Hercules variety**	32.90 ±0.08 de	171.72 ±0.25 j	28.82 ±0.12 g	145.54 ±0.22 j
13	**CO_2-extract hops of the Hercules variety**	35.42 ±0.09 f	185.32 ±0.32 k	29.34 ±0.10 g	149.92 ±0.16 k
	minimum	27.33	171.72	20.96	145.54
	maximum	36.00	281.61	30.79	222.63
	mean	31.21	220.23	26.98	186.44

Note: *value ±SD are means of three repetitions, *Mean values followed by different letters are statistically different at p <0.05.

Hops and hop products added at the rate 50 mg of bitter substances per 1 dm^3 of wort. However, the taste of beer and the quality of bitterness in different samples were different. As indicated results researches of biochemical composition, the reason of this difference in the content of bitter substances and other components in the varieties of hops and hop products that added to wort. At the beer boiled added various hops products with the same content of alpha acids but different content of other valuable brewing components of hop. The quality of beer which was produced by one technology, but using different types of hop products was significantly differ. Our studies were correlation with other researches of the domestic and foreign scientists **(Lyashenko, 2002; Sheiko et al., 2019)**, which established that beer made from hops of various varieties was significantly different in character of bitterness, taste and aroma. The reason of this the peculiarity of the biochemical composition of bitters substances, polyphenolic compounds and essential oils of hop of aromatic and bitter varieties. Different ratio of components of these compounds differently affects on the taste and aromatics of beer. With taking into account, for determine the suitability of hops and hop products for beer making need carry out their complex comprehensive technological assessment of biochemical composition of the hops products.

According to the results of the tasting (Table 3), all samples of beer had a pleasant fresh beer flavor. The hop flavor was well presented in the beer with hopped wort by cones of pressed hop and pellets of hop type 90 varieties Slavyanka, Nationalny, Zagrava and Alta. In the samples of beer made from ethanol extract and CO_2-extract of hop of the Hercules variety, the aroma of hops was almost absence. The beer made from cones of pressed hop and pellets of hop type 90 varieties Slavyanka and Nationalny for bitterness and taste quite similar to each other. The bitterness was very light, delicate, soft, but in the beer made from pellets of hop type 90 varieties Slavyanka and Nationalny somewhat excess. The taste of this beer was full, harmonious. Beer obtained by adding to the wort pressed cones and pellets of type 90 of hop variety Zagrava for the quality of bitterness and taste similar. The beer has little harmonious taste and delicate, pleasant, balanced bitterness. But the bitterness of beer with added to the wort pellets type 90 of the Zagrava variety was more intense. When added to the wort pressed cones of hop of the Alta variety, pellets of hops type 90 varieties Alta and Hercules beer had rough bitter. Fullness of taste not detected.

Table 3 Organoleptic (tasting) evaluation of beer, depending on the characteristics of the hops and hop products, points.

No.	Grade of beer Variant of the experiment	Names of quality indicators: Taste: Transparency	Colour	Foaming	Flavour	fullness	hop bitterness	Total evaluation, points	Evaluation
1	Cones of hop of the Slavyanka variety	3.0	3.0	5.0	3.7	4.5	4.4	23.6	excellent
2	Cones of hop of the Nationalny variety	3.0	3.0	5.0	3.8	4.0	4.2	23.0	excellent
3	Cones of hop of the Zagrava variety	3.0	3.0	5.0	3.4	3.9	4.0	22.3	excellent
4	Cones of hop of the Alta variety	3.0	3.0	5.0	3.5	3.5	3.3	21.3	well
5	Pellets of type 90 Slavyanka variety	3.0	3.0	5.0	3.9	4.2	4.3	23.4	excellent
6	Pellets of type 90 Nationalny variety	3.0	3.0	5.0	3.8	4.2	4.2	23.2	excellent
7	Pellets of type 90 Zagrava variety	3.0	3.0	5.0	3.6	3.9	4.1	22.6	excellent
8	Pellets of type 90 Alta variety	3.0	3.0	5.0	3.5	3.5	3.5	21.5	well
9	Pellets of type 45 Tradition variety	3.0	3.0	5.0	3.7	4.0	4.0	22.7	excellent
10	Pellets of type 45 Spalt Select variety	3.0	3.0	5.0	3.8	4.1	4.5	23.4	excellent
11	Pellets of type 90 Hercules variety	3.0	3.0	5.0	2.2	2.9	2.9	19.0	well
12	Ethanol extract hops of the Hercules variety	3.0	3.0	5.0	3.0	3.3	3.5	20.8	well
13	CO_2-extract hops of the Hercules variety	3.0	3.0	5.0	3.1	3.0	3.4	20.5	well

The beer made from pellets of hop type 45 varieties Tradition had bound, balanced bitterness with a pleasant hops aroma. Adding to the wort the pellets of the hops type of the 45 variety Spalt Seleck provided the beer with fresh hop flavor, full harmonious taste and bound, pleasant, balanced bitterness.

Beer produced for the ethanol and CO_2-extracts of the Hercules variety of taste and bitterness were almost indistinguishable. The bitterness was rough, exceed. Beer was empty, without fullness of taste.

CONCLUSION

Complex researches of hops and hop products of various varieties with used of modern biochemical methods allow was established that hops and hop products of different varieties have different biochemical composition and hence difference brewing value. Differences depend on the absolute values of such parameters as the mass fraction of α-acids, β-acids and their composition, xanthohumol, general polyphenols, essential oils, the ratio of their valuable components of hops: β-acids to α-acids, and also the general quantity of polyphenols and essential oils per unit of α-acids.

Hops pellets of type 90 of domestic production contain the whole complex of necessary for brewing substances which were equivalent to hop cones. Characteristic feature of pressed cones of hop and hop pellets of type 90 and type 45, in particular of aromatic varieties, were high positive coefficient of aromatics between the content of β- and α-acids, ranging from 0.9 till 1.8. In opposite in pellets and extracts in hop cones presented more essential oils per 1 g of α-acids, which provides obtained more aromatic beer.

Hops pellets of the type 45 of foreign production enriched in the content of α-acids in their composition contained less amount of essential oil than hop cones and hop pellets type 90, which associated with the technology of obtaining pellets of this type.

Ethanol and CO_2 extracts had concentration of α-acids up to 50 % or more, which ensured the benefits of these products during storage, transportation and using in brewing. But these extracts do not had the required quantity of polyphenolic compounds of hops necessary for the normal implementation of the brewing process and obtaining full-fledged beer. They contained less quantity of essential oil, but it did not enough for the optimal ratio with alpha acids. Therefore, when making

beer, it is necessary to add certain quantity of cones or hops granules.

Technological assessment of the selection varieties of pressed cones of hop and hop products showed that all presented thin-aromatic and aromatic hops types Slavianka, Nationalny, Zagrava and pellets type 90, made from them and also pellets of type 45 varieties Tradition and Spalt Select were suitable both for self-use in brewing and for improve the taste of beer in combination with other processed products. Beer made from hops pellets, especially the Zagrava variety, had an excess of bitterness, therefore to norm pellets for beer production due to with an economy of up to 10 %. The individual used of pressed cones of hop and pellets of the bitter variety Alta and Hercules does not allow obtained the bitterness of beer of excellent quality. Ethanol and CO_2 extracts for self-use in brewing were not suitable. It was possible to recommend their using in combination with cones and pellets of aromatic varieties, while adhering to certain technology.

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THE INFLUENCE OF LIFESTYLE ON CONSUMER BEHAVIOR AND DECISION MAKING IN RESEARCH AIMED AT PROTEIN BARS

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ABSTRACT

We live in an era when all circumstances on the market are changing rapidly, which leads consumers (even, if they are not aware of it) to certain behaviour that affects their daily activities. Lifestyle can be described as someone's way of living or the things that a person or a particular group of people usually do. It is included among the modern elements of consumer behaviour and also affects an individual´s decision-making process. The concerns over obesity and dangerous food ingredients have prompted a "healthy lifestyle" to become the latest trend in marketing. Therefore, the regular exercising, the reduction of stress, drinking enough water, and eating nutritious food takes on its importance. The main objective of the paper is to assess the consumer behaviour on the market of a selected food commodity. For this purpose, protein bars, which are part of diet not only of athletes but also of ordinary consumers, have been chosen. To achieve this main goal, a questionnaire was designed and data were collected from the respondents of the different age groups in the Slovak Republic. Based on the primary results, the authors of the paper can claim that more than 60% of the respondents try intentionally to choose better options of food as they want to live healthy. For a deeper analysis, the assumptions were formulated and subsequently verified by the Pearson Chi-square test of independence and Kolmogorov-Smirnov test. The paper provides useful information on consumer behaviour that can help not only producers and retailers but also to consumers themselves.

Keywords: consumer; consumer behavior; lifestyle; food market; protein bar

INTRODUCTION

Recently, the psychosocial aspects of health have held interest. The constructs such as health perception and health-related quality of life are included in a broader domain because they pertain to positive health **(Grao-Cruces, Fernandez-Martinez and Nuviala, 2014)**. Individuals frequently face a "want-should" conflict between taking unhealthy food or activities and maintaining a healthy lifestyle. Such conflict is in nature a trade-off between a smaller immediate reward and a larger delayed reward **(Van Beek et al., 2013)**. An immediate oriented individual would choose an immediate reward over longterm benefits and thus make a less healthy decision **(Li and Hu, 2019)**. HA healthylifestyle includes taking physical exercise, keeping dietary eating and quitting smoking **(Adams and Nettle, 2009; Joireman et al., 2012)**. There are many benefits of the healthy lifestyle. It can increase a person's resilience and mental well-being **(Cairns et al., 2014)**. Understanding the factors that contribute to the healthy lifestyle behaviour is critical to the development of the interventions needed to promote the positive behaviour that can prevent the negative physical and mental health outcomes, which may have lifelong implications **(McGovern et al., 2018)**.

Lifestyle, but what is more a dietary pattern and has been changed rapidly by the development of urbanization, industrialization of societies and increased economic growth in the last decades **(Bolori et al., 2019)**. According to the World Health Organization, a healthy diet contains fruit, vegetables, legumes, nuts and whole grains, while also containing the limited amount of free sugars, salt and fat, and an amount of calories, which is in balance with energy expenditure **(World Health Organization, 2018a)**. By **Forshee et al. (2018)** the diets rich in fruits and vegetables and low in sugary foods and drinks were found to be associated with a lower-body mass index (BMI) **(Mytton et al., 2014; Ebbeling et al., 2012)**, but over 1.9 billion adults worldwide were overweight in 2016, resulting in higher prevalence of chronic diseases such as cardiovascular disease and diabetes **(World Health Organization, 2018b)**. In rodent models, changing the typical diet to one characterized by high-saturated fat, salt, and sugar consumption (often referred to as a"Western diet") reliably increases abdominal fat, insulin resistance,

atherosclerosis, and inflammation. Consuming this type of diet, has been linked to chronic, low-grade inflammation and associated diseases such as cancer, heart disease, and diabetes **(Christ et al., 2018; Huang et al., 2013; Thorburn et al., 2014)**.

High-protein foods are popular among the consumers seekingsatiety, increase muscle mass, or decreased risk of sarcopenia **(Sloan, 2012)**. Hence, protein bars have received growing attention in sports nutrition, musclebuilding, health supplement and weight reduction markets in recent years **(Kelly et al., 2019)**. The consumers are turning to the high-protein nutrition bars to add conveniently more protein to their diet. The high-protein nutrition bars have used new, trendy protein sources (e.g., insects), but have traditionally relied on dairy and soy ingredients such as concentrates, isolates, and hydrolysates **(McMahon et al., 2009; Imtiaz et al., 2012)**. These bars contain mainly protein (20 – 50%), carbohydrate (e.g. high-fructose corn syrup), fat (e.g. palm oil) and some humectants with a water activity of 0.5 – 0.8.

Scientific hypothesis

Assumption No. 1: We assume that there is no relationship between particular sex and the purchase of healthy food.

Assumption No. 2: We assume that there is no dependence between the respondent's sex and how often they engage in the sports activity.

Assumption No. 3: We assume that there is a relationship between particular sex and the consumption of protein bars.

Assumption No. 4: We assume that there is no correlation between the respondent's age and the preferred flavor of protein bars.

Assumption No. 5: We assume that there are differences between the influence of individual factors on the purchase of protein bars.

MATERIAL AND METHODOLOGY

The methodology of the paper was constructed through a questionnaire survey. The implementation was carried out both physically and using the Google Forms platform. The questionnaire survey was carried out in the Slovak Republic. The survey sample consisted of 627 respondents. The age structure was focused on the whole population. The survey was divided into three parts. The first part dealt with the demographic structure, the second one was generally focused on the respondents' lifestyle. The third part examined the consumer behavior of the selected food commodity. The main objective of the paper is to map a certain lifestyle of the respondents. The survey was used to find out consumers` behavior, preferences and decisions in everyday activities – buying of the selected food commodity.

The basic demographic data are given in Table 1. As far as the economic status is concerned, most surveyed are students (60.29%), followed by the respondents who are employed (30.94%). Almost half of the respondents (49.92%) completed the secondary education with the school-leaving examination as the highest achieved education, followed by students of grammar schools (18.98%) and then university graduates (15.95%).

Table 1 Characteristics of respondents.

Category of Respondents	%
Male	40.83
Female	59.17
Place of Residence	**%**
City	47.21
Village	52.79
Age Structure	**%**
Less than 20 years	47.21
21 – 30 years	34.45
31 – 40 years	4.94
41 – 50 years	9.09
51 years and more	4.31
Number of household members	**%**
1 – 2	16.75
3 – 4	62.68
5 and more	50.75

Statistical analysis

Before the questionnaire investigation, the scientific assumptions were established, whose testimony was verified by the means of a selected statistical method – Pearson Chi-square test of good conformity and Kolmogorov-Smirnov test. The authors of the paper have also used pivot tables whose application was realized on the obtained primary data. The authors determine the probability level - alpha ($\alpha = 0.05$), which will be compared with the level of significance (p-value). Based on alpha (α) it is possible to evaluate the hypothesis with a comparison of the p-value. If the p-value is less than alpha (α), we reject H_0. If the p-value is greater than alpha (α), we do not reject H_0.

RESULTS AND DISCUSSION

627 respondents participated in the questionnaire survey. The primary data were obtained to a greater extent from women (59.17%). In this survey 256 male respondents (40.83%) took part. The respondents were divided into five groups based on their age. The largest representation was the age structure under 20 years (47.21%). This was followed by an interval from 21 to 30 years (34.45%). These two age categories were the most important for the authors of the paper as we assume that the consumption of protein bars in the above age structures is the greatest. The age range from 41 to 50 years was 9.09%. Almost half of the respondents cited as the highest education a secondary school with a school-leaving certificate (49.92%). This was followed by grammar school leavers (18.98%) and university graduates (15.95%). The most frequently reported economic status was a student (60.29%). The second most frequently chosen option was the economic status - employed (30.94%). The authors of the paper also considered the family status, more than 2/3 of the respondents declared they were single. Only 14.51% of the participants stated that they were married. The last and also a very important question of the demographic survey was the net monthly income of the respondents (Figure 1).

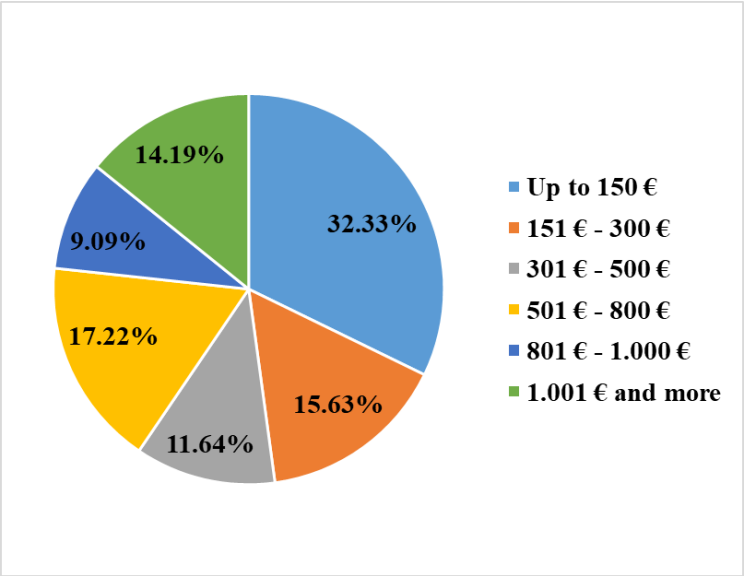

Figure 1 Monthly income of respondents.

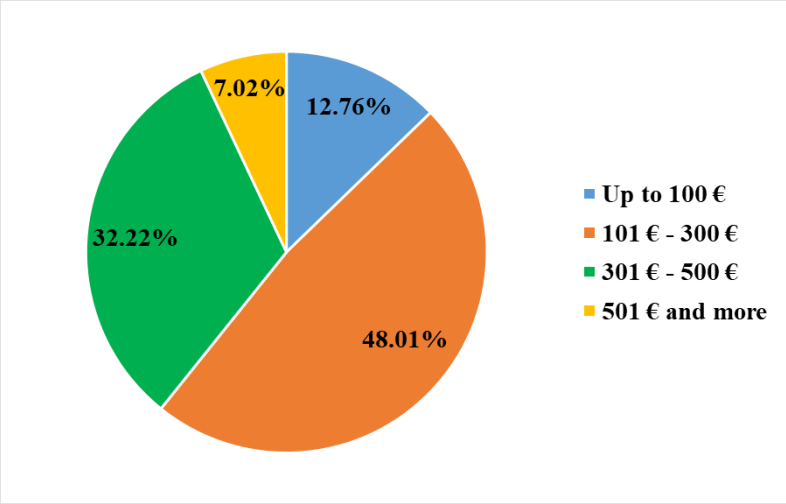

Figure 2 Monthly food expenses.

It follows from Figure 1 that the biggest interval up to 150 € constituted 32.33%. This was mainly because the maximum age limit was up to 20 years, and therefore, this age group does not have a big income opportunity. In the second place, there was an income from 501 € to 800 € with a percentage of 17.22%. An interesting feature of this survey, however, is the income interval above 1.001 €, which marked 14.19%. This was followed by a group of questions that dealt generally with consumer purchasing behavior. In this group of questions there were also formulated questions about the lifestyle of respondents. The authors of the paper have also considered the preferences and individual consumer impact factors. The first question focused on buying daily food consumer frequency. The observed values are given in Table 2.

The table shows that the respondents most often do shopping once or twice a week (42.11%). The second most frequent interval (three to four times a week) was 41.74%. Interestingly, up to 64 respondents buy daily groceries every day (10.21%).

Table 2 Frequency of shopping.

Frequency of shopping	Count	%
1 – 2x per week	264	42.11
3 – 4x per week	260	41.74
5 – 6x per week	39	6.22
Every day	64	10.21

Within this group of questions the authors of the paper have asked the respondents what was the decisive factor of their choice when buying food. In this case, the respondents identified quality as the most important factor (62.04%). Quality was also critical in surveys by **Grunert (2005)**. In the second place there was the price (25.68%), which is constantly placed in the leading position among the influencing factors on consumer purchasing decisions **(Gilbert, 2010; Ubreživá et al., 2019)**. This was followed by the origin of the particular foodstuff purchased (5.90%) and finally in the last place there was the package size (3.35%). The remaining 3.09% was divided among several factors that consumers had to choose (design, hunger, smell, composition, etc.).

This was followed by the related question, how much on average the whole family spends on food per month. The data are shown in Figure 2.

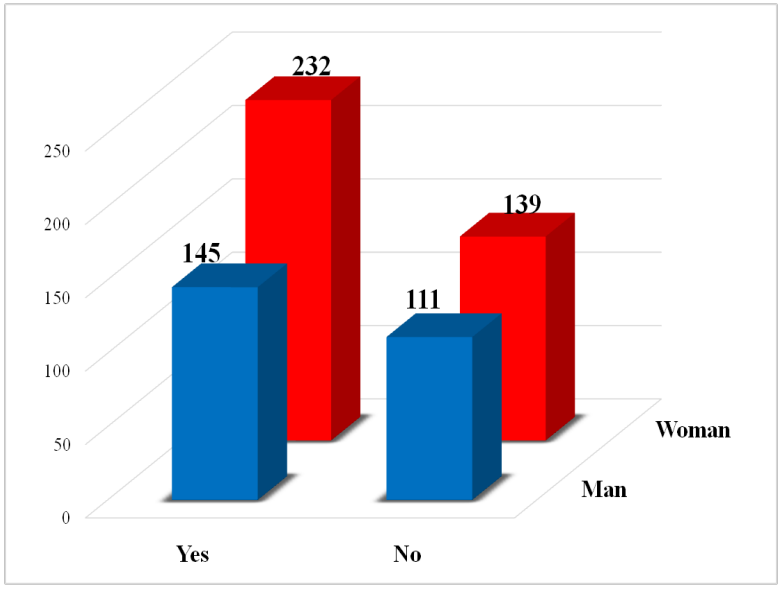

Figure 3 Confirmation of statistical assumption.

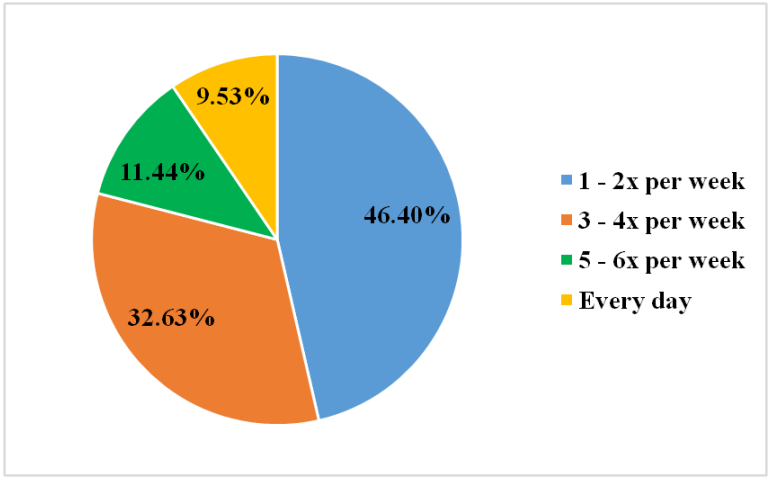

Figure 4 Sport activity.

From Figure 2, it is clear that the largest interval a family gives monthly to buy food is from 101 € to 300 €. Almost half of the respondents (48.01%) chose this option. Furthermore, the interval of 301 € to 500 € occurred. Spends only 7.02% spends on food more than 501 €.

Furthermore, the authors of the paper have concentrated their attention on respondents' lifestyles. They were asked if they were deliberately buying healthy food. Most of the respondents stated that they were deliberately buying healthy food (60.13%). 250 respondents (39.87%) answered negatively. If the respondents replied´yes´, they were asked what was the reason for such a purchase. More than half (51.89%) of the respondents stated that they want to live more healthy as a reason for purchasing **(Sjöberg et al., 2003; Kubicová and Habánová, 2012)**. The second most common answer was the possibility of self-belief (39.04%). Health reasons took last place (9.07%).

Based on the factors, the statistical observation was made on the presumption - whether there is a relationship between sex and the purchase of healthy food.

H_0: There is no relationship between sex and the purchase of healthy food.

H_1: There is a relationship between sex and the purchase of healthy food.

The *p*-value of Pearson's Chi-square good-fit test is used to verify the hypotheses. Testing will be carried out at the selected level of 5% significance, i.e. alpha will be 0.05. In this case, the *p*-value = 0.0941, which means that we reject the null hypothesis. We accept the alternative hypothesis and declare that 95% reliability is the relationship between sex and the purchase of healthy food. Based on the test results, the assumption is incorrect. Subsequently, the authors of the paper have also presented the results in Figure 3, which confirms the statistical testing.

Figure 3 shows that women buy healthy food more often. In general, women buy more often than men **(Beardsworth et al., 2002; Géci et al., 2017)**. Men in this case buy much less healthy food than women do.

Then, the respondents were asked whether they considered their lifestyle as healthy. Most respondents consider their lifestyle to be healthy, 74.80%. The remaining 158 respondents are not interested in a healthy lifestyle. For better clarity, we have made a comparison based on gender. The above question shows that both genders have the same values (positive and negative) when

compared. The female sex lives healthy at 74.66% and the male sex at 75.00%. This was followed by a related question, which dealt with the reason why a healthy lifestyle is a part of their lives. The vast majority responded that their own beliefs (84.58%) were a factor in a healthy lifestyle. **Moreno et al. (2008)**, achieved almost identical results. Then it was followed by family and friends (8.13%) and finally various health problems (7.29%).

A certain type of exercise (sports activity) is also closely related to a healthy lifestyle. Therefore, the authors of the paper have asked the respondents, if they were doing any sport (active or passive at least twice a week). 69.70% of the respondents gave a positive answer to the question. The authors have wondered how often they practice sport activities (Figure 4).

More than 46% of respondents claimed that they practice sport activities once or twice a week. More often, 32.63% carries out sporting activity. Interestingly, 45 respondents reported daily physical activity.

Based on the factors, the statistical observation was made on the presumption - whether there is a dependence between gender and frequency of sports activity.

H_0: There is no relationship between gender and frequency of spsportsctivity.

H_1: There is a relationship between gender and frequency of sports activity.

To verify the established hypotheses, the authors of the paper have used the *p*-value of the Kolmogorov-Smirnov test of good conformity. Testing was carried out at the selected level of 5% significance, i.e. alpha as 0.05. In this case, the *p*-value = 0.0154, which means that we reject the null hypothesis. We accept the alternative hypothesis and declare that 95% reliability is the relationship between the sex and frequency of sporting activity. Based on the test results, the authors of the paper have considered the assumption to be correct.

Finally, the authors came to the researched issue, which is a consumption and consumer behavior when buying a selected market commodity - protein bars. For this group of questions, the authors of the paper mainly deal with the reason and time of consumption, the preferred flavor, and the factors of choice for this food snack.

The first and main question of this section was concerning the consumption of protein bars (Table 3).

The authors of the paper have asked whether the respondents generally consume protein bars.

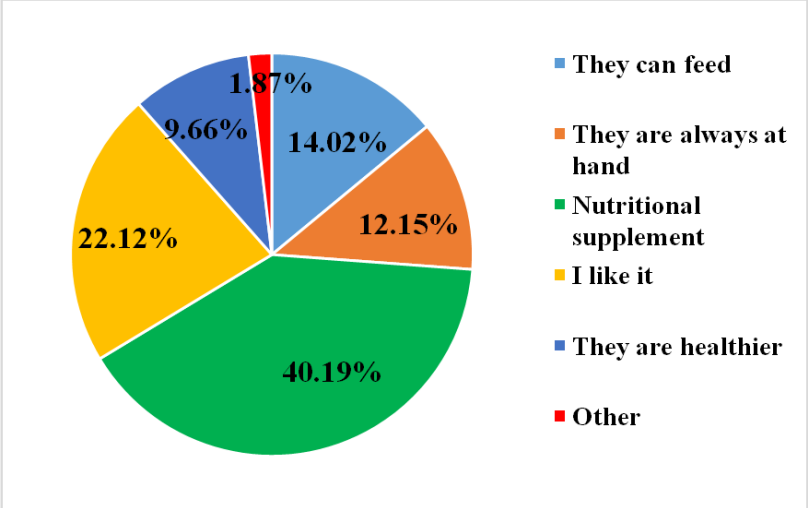

Figure 5 Reasons for consumption.

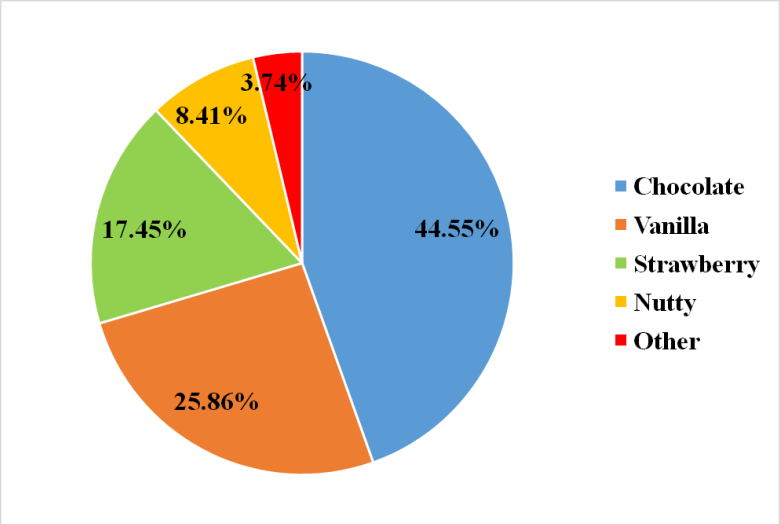

Figure 6 Preferred flavors.

Table 3 Consumption of protein bars.

Consumption	Count	%
Yes	321	51.20
No	306	48.80

The table clearly shows that the majority of the respondents do not consume the selected food commodity (51.20%). We have assumed such a result as protein bars are specialized food and not everyone knows and looks for this kind of food. Here the survey for some respondents is over. The authors of the paper have filtered out the respondents who do not consume protein bars so that testing can continue to evaluate the primary data obtained. From the original number of 627 respondents, only 321 respondents continue to be evaluated.

Based on the factors, the statistical observation was made on the presumption - whether there is a relationship between a sex and protein bar consumption.

H_0: There is no relationship between sex and protein bar consumption.

H_1: There is a relationship between sex and protein bar consumption.

The *p*-value of Pearson's Chi-square good-fit test is used to verify the hypotheses. Testing was carried out at the selected level of 5% significance, i.e. alpha as 0.05. In this case, the *p*-value = 0.0257, which means that the authors of the paper have rejected the null hypothesis. The authors accept the alternative hypothesis and declare that p 95% reliability is the relationship between sex and protein bar consumption. Based on the test results, the authors of the paper consider the assumption to be correct.

This was followed by a question about the frequency of protein bar consumption among the respondents. Most respondents (46.73%) declare they only consume protein bars occasionally. In this case, this means that eating this food does not have a regular place in the respondents' menus. The consumption was admitted once or twice a week (38.94%). In this case, the authors of the paper can assume that such consumers engage in some kind of sports activity. The last most frequently reported option was an interval of three to four times a week (11.21%). The authors of the paper have discussed the reason for buying this food commodity. This type of food belongs to specific and low-demand food for the majority of the population. The reasons for buying this specific type of food are shown in Figure 5. The chart shows that the respondents consume protein bars based on their nutritional value (40.19%). In this case, however, not all protein bars have the appropriate nutritional values **(Fernan et al., 2017)**. The research has evaluated and tested this type of food, sensory testing or, laboratory testing for these ingredients and nutritional values. It was concluded that not each protein bar should be called a protein bar. Some of them contain much more sugar than proteins and in this case they should not be categorized as protein bars **(Imtiaz et al., 2012; Zhou et al., 2013)**. The original protein bar should contain as little sugar as possible and the protein content should exceed the sugar value **(Li et al., 2008)**. The taste came in the second (22.12%) as the reason for the choice. As part of this response, the respondents declare they like protein bars and therefore buy them. In this case, they do not pay attention to the reasons for purchasing this specific commodity. 39 respondents said they buy protein bars mainly because they are always on hand when they get hungry. With this possibility, however, it should be kept in mind that protein bars are only a nutritional supplement **(Maughan et al., 2007)**. They do not replace a full-fledged and mainly nutritious meal in any way**(Singh et al., 2008)**. In the last place (1.87%) there were various other reasons for the consumption and purchase of protein bars, such as consumption is fashionable.

The authors of the paper have also asked the respondents when they consume protein bars most often. So whether they have a daily ritual of consuming these special kinds of food or do not solve it during the day. Most respondents stated that they do not need any special reason to consume sticks, and therefore they consume them at any time of the day when they feel like eating it (53.89%). Then followed the possibilities of consumption, which are already a daily ritual. The second most marked place was consumption after sports activity (31.46%). This was followed by consumption before sports activity (9.35%). According to **Corrado et al. (2003)**,sportst activity refers to any physical activity related to sport (training, exercis,e and various types of sport). In this case, the consumption before and after sports activity is due to the nutritional composition of various substances that have a positive effect on the stressed body **(Thiansilakul et al., 2007)**.

Another essential question of protein bar consumption was the question of preferred protein bar flavor. These flavor preferences are shown in Figure 6.

It follows from Figure 6 that the respondents prefer the consumption of chocolate protein bars (44.55%). Vanilla flavor (25.86%), strawberry flavor (17.45%), and hazelnut flavor (8.41%) followed. In the last place, there was the possibility of other flavors (3.74%). In this case, the respondents had in mind various other flavors such as lemon cake, coconut flavor, or various other fruit flavors in the limited editions.

Based on the factors, the statistical observations were made on the assumption - whether there is a relationship between age and the preferred flavor of protein sticks.

H_0: There is no relationship between age and preferred flavor of protein bars.

H_1: There is a relationship between age and preferred flavor of protein bars.

To verify the established hypotheses, the authors of the paper have used the *p*-value of the Pearson Chi-square test of good conformity. Testing was carried out at the selected level of 5% significance, i.e. alpha as 0.05. In this case, the *p*-value = 0.0358, which means that the authors have rejected the null hypothesis. The authors of the paper have accepted the alternative hypothesis and confirm that 95% confidence is the relationship between age and preferred flavor of protein bars. Based on the test results, the authors of the paper have considered the assumption to be correct. For better clarity, see figure 7.

It is clear from Figure 7 that the age structure influences the flavor decisions. This was followed by a question about the choice of protein selection factor. The respondents had a choice of five factors affecting their purchase. These factors were arranged from the most important to the least important factor. The numbers from 1 to 5 were assigned to each factor, with a score of 1 being the least important factor and the value of 5 being the most important factor. These values are shown in Figure 8 for clarity. The figure shows that the most important factor in the selection of protein bars is their particular flavor. Up to 45.18% of the respondents claimed the importance of the flavor. The flavor itself is a very important factor in the choice of any food **(Guichard, 2007)**. Interestingly, the selection factor nutritional supplement – 25.00% ended in the second place. This was followed by a brand, which is one of the basic factors for choosing a particular product. Subsequently, the price was placed (9.04%) and packaging was the least important factor. In very few cases the packaging itself is a decisive point in the choice of food **(Pollard et al., 2002; Pierański et al., 2017)**. This is due to the saturation of the market for this food commodity, as well as due to its specific location and respondents' attitude to its consumption **(Wells et al., 2007; Kozelová et al., 2014)**.

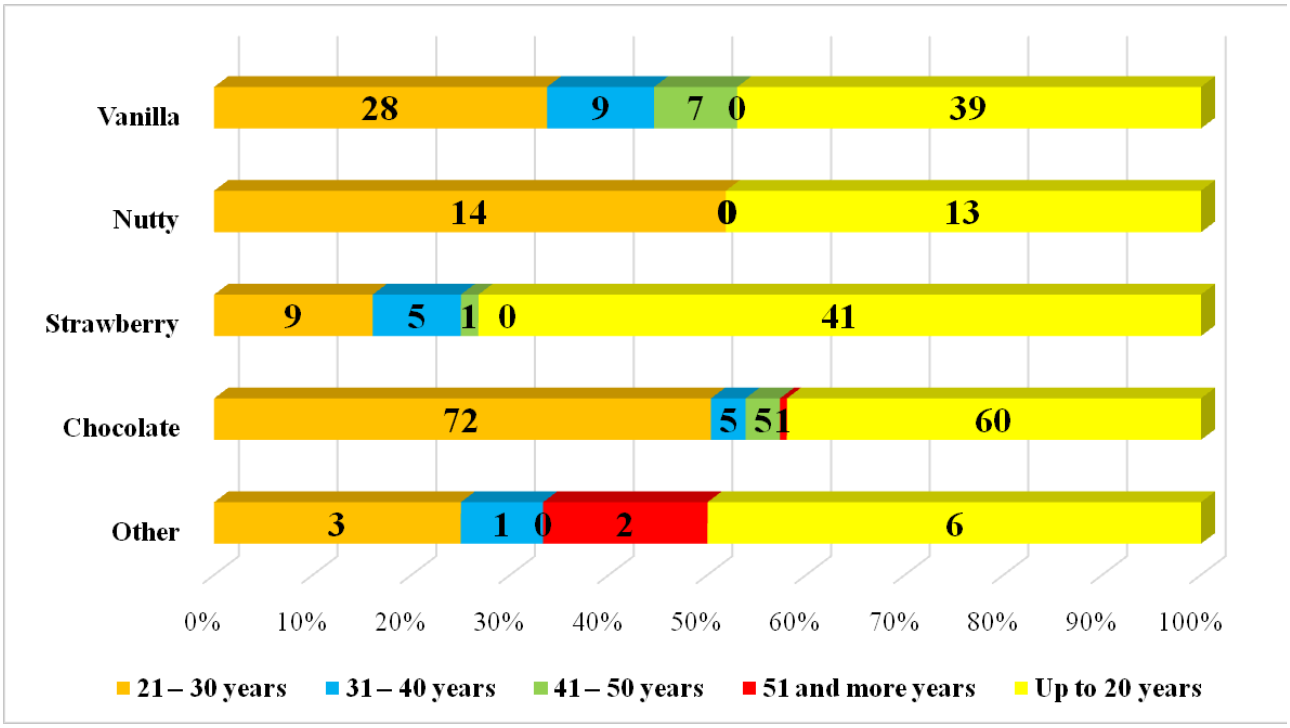

Figure 7 Preferred flavor by age.

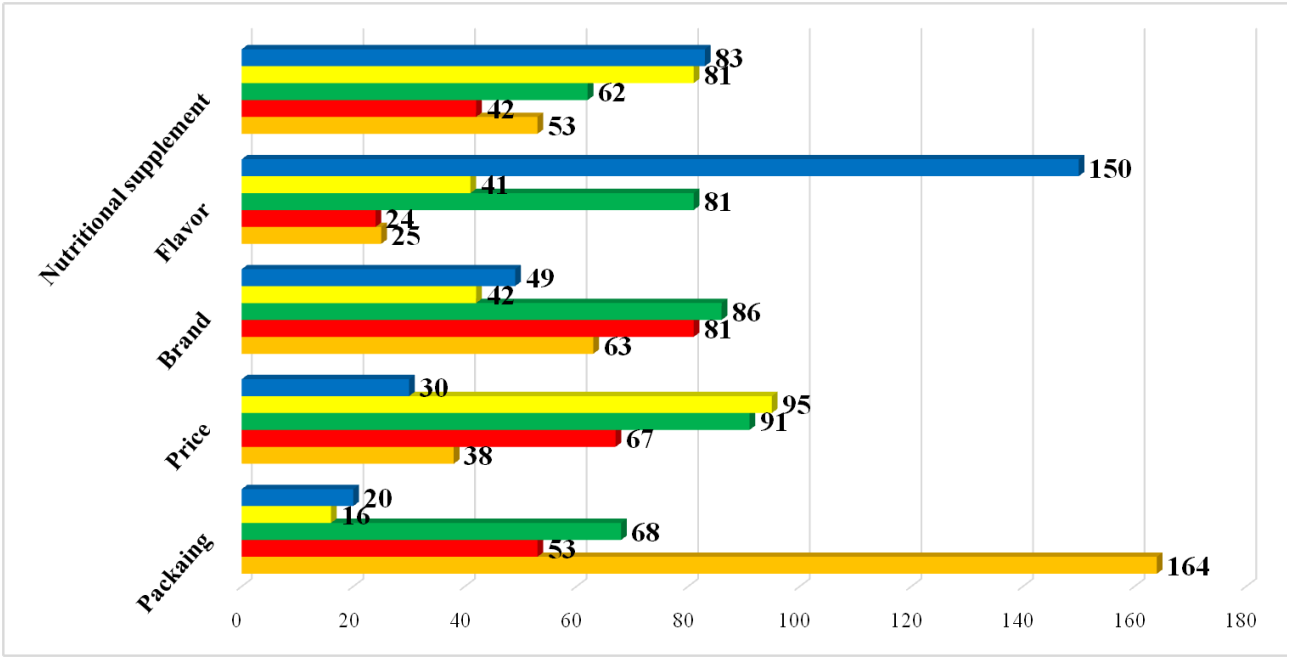

Figure 8 Protein flavor selection factor.

Based on the factors, the statistical observations were made on the assumption - whether there are differences between the influence of individual factors and the purchase of protein bars.

H_0: There are no differences between the influence of individual factors and the purchase of protein bars.

H_1: There are differences between the influence of individual factors and the purchase of protein bars.

The authors of the paper have used *p*-value from the Kolmogorov-Smirnov test of good conformity to verify the hypotheses. Alpha will be 0.05. In this case, the *p*-value = 0.0158, which means that the authors of the paper reject the null hypothesis. The authors accept the alternative hypothesis and declare that with 95% reliability, there are differences between the influence of individual factors and the purchase of protein bars. Based on the results, the authors of the paper consider the assumption to be correct.

The last question of the questionnaire survey focused on one of the most important factors, which plays an essential role in the purchase of food – and that is a price. The authors of the paper asked the respondents how much they are willing to spend on a protein bar. General weight of 100 grams was set for this question. Most respondents chose between 1.1 € and 2 € (73.52%). In the second place, there was a sum from 2.1 € to 3 € (17.76%). 6.85% of respondents indicated the price value up to 1€. In the last place, there was an interval of more than 3 €, which was chosen only by 6 of the surveyed consumers.

CONCLUSION

The paper analysed the consumer behaviour on the market of the selected food commodity – protein bars, which can be classified as healthy food. The survey was carried out on the selected sample of the respondents with the different gender, age, and lifestyle to be in accordance with the main sample of citizens of the Slovak Republic. The results showed that more and more consumers are becoming interested in the quality of products they purchase, as up to 62.04% of respondents stated the quality as a decisive factor when purchasing food and the price, which has always been at the forefront of the factors, reaching the second place (25.68%). The survey focused also on the healthy lifestyle of the respondents and buying healthy food. In this case, more than half of the respondents stated that they intentionally buy healthy food (60.13%), as they want to have healthy, full-featured, and longer life (51,69%). As far as the healthy lifestyle is concerned, up to 74.80% of the respondents were convinced that they can describe their lifestyle as healthy, and more than half of them reported practicing sport activities once or twice a week. Subsequently, the questions related to the purchase and consumption of protein bars followed. The most responses (51.20%) do not choose this food commodity, however, these results were expected as the protein bars are classified as specific food. The respondents, who purchase them, do it for their nutritional value (40.19%), but paradoxically they choose among them according to the flavor (45.18%) and not nutritional value (25.00%), and only 31.46% of the respondents consume this type of food after sports activity.

When buying the different food commodities, the consumers behave individually, and therefore, there is no general formula that can describe fully this daily shopping behaviour. Ultimately, the authors of the paper can conclude that consumer behaviour is unpredictable in all respects and the marketers can just work with their assumptions.

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ISOLATION AND IDENTIFICATION OF ACTIVE COUMPOUND FROM BENGLE RHIZOME (*ZINGIBER CASSUMUNAR* ROXB) AS A STIMULANT IN PHAGOCYTOSIS BY MACROPHAGES

Muhamad Fauzi Ramadhan, Nurkhasanah Mahfudh, Nanik Sulistyani

ABSTRACT

Immunomodulators are pharmacological agents that modify or regulate the immune system through stimulating the functioning of the immune system and, at the same time, inhibiting excessive immune responses. This study was conducted to determine the active compound in *Z. cassumunar* that is responsible for increasing the immune system based on the parameters of phagocytic activity. The isolation method began with fractionation, which involved extraction with ethanol and successive fractionation with hexane and chloroform. *Z. cassumunar* extract, hexane fraction, and chloroform fraction were tested on mice macrophage cells for their phagocytic functions. The phagocytic activity of macrophages was measured by active phagocytic cells (averagely 39.194 ±1.597, 27.923 ±2.941, and 62.090 ±6.947) and phagocytic index (in a row, averagely 47.513 ±2.844, 41.129 ±7.195, and 101.527 ±10.555). The results showed that the *Z. cassumunar* extract, hexane fraction, and chloroform fraction exhibited more significant phagocytic activities of macrophages (p <0.05) compared with the normal group. Since the chloroform fraction showed the best result, this fraction was further separated by column chromatography. This procedure yielded five sub-fractions, namely F1, F2, F2C, F3, and F4. Based on the phagocytic activity testing, the results were as follows: (1) the active phagocytic cells of F1, F2, F2C, F3 and F4 were 18.860 ±3.191, 27.077 ±4.482, 15.749 ±3.026, 64.333 ±1.780, and 44.943 ±2.944, respectively, and (2) the phagocytic indices were 30.0249 ±3.4231, 44.5969 ±8.3646, 24.5597 ±5.4487, 102.7447 ±1.0806, and 76.5007 ±4.7293. Because F3 produced the best result, this subfraction was then identified using 1H-NMR and 13C-NMR. The identification results showed that F3 was (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol as an active compound.

Keywords: *Zingiber cassumunar*; phagocytosis macrophage; (E)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol

INTRODUCTION

Through various studies on effects and efficacy, traditional medicine has been increasingly developed for its use in overcoming health problems. Around 59.1% of the Indonesian population use traditional medicine, and 95.60% of which claim to perceive the benefits. This figure is supported by the abundant natural resources in Indonesia and the willingness to start following WHO's recommendations to incorporate natural ingredients in treatment and lifestyle. To substitute for immune enhancers, people tend to prefer traditional medicine **(Indonesian Health Ministry, 2013)**. The immune system consists of organs, tissues, cells, and chemical compounds that are used by the body to protect it from the danger of foreign objects or cells that potentially cause abnormal internal changes. The immune system is divided into two types, namely specific and non-specific. The non-specific immune system is the body's first defense against the attacks of foreign bodies because it responds directly to antigens. whereas, a specific immune system is a system that can recognize foreign substances that enter the body and can stimulate the development of specific immune responses to these substances **(Baratawidjaja and Rengganis, 2014; Hooper, Littman, and Macpherson, 2015; Shiland, 2014)**. In this system, macrophages play the most crucial role. They act as effectors on therapeutic bodies in the immune system against pathogens using the phagocytosis mechanism, either directly or indirectly, by releasing ROI and cytokines. These functions can be influenced by immunomodulators **(Baratawidjaja and Rengganis, 2014; Gabrilovich and Nagaraj, 2009)**. Immunomodulators are drugs that enhance immune systems with low function (immunostimulants), suppress excessive immune function (immunodepressant), and increase vaccine efficacy (Immunoadjuvant). In other terms, immunomodulators help the body to optimize the role of its immune system. One of the natural ingredients that have the potential to modulate the immune system is *Z. cassumunar* **(Baratawidjaja and Rengganis, 2014; Bascones-Martinez et al., 2014; Chairul, 2009; Nair, Chattopadhyay, and Saha, 2019)**.

Z. cassumunar is a type of rhizome from the Zingiberaceae family. It contains several chemical compounds, namely essential oils, starch, resin, tannins, flavonoids, and phenylbutanoic acids. This rhizome is often used as a medicine to treat various diseases, including fever and headache, and to increase immunity **(Hartati et al., 2013; Utami, 2008)**. In previous research, *Z. cassumunar* has been reported as an effective adjunctive therapy in malaria **(Hermansyah and Utami, 2015)**.

Several studies have succeeded in isolating phenylbutanoid derivatives from *Z. cassumunar*, namely (E) -4- (3 ', 4'-dimethoxyphenyl) but-3-en-1-ol; (E) -4- (2 ', 4', 5 '-trimethoxyphenyl) but-3-en-1-ol; (E) -4- (3 ', 4', 1-trimethoxyphenyl) but-3-en-1-ol; (E) -4- (3 ', 4'-dimethoxyphenyl) but-3-en-1-il-acetate, and methoxy-8-(3,4-dimethoxyphenyl) -1,4-napthoquinone **(Chairul, 2009; Hartati et al., 2013; Kuroyanagi et al,. 1980)**.

Scientific hypothesis

Z. cassumunar has compounds that are responsible for the phagocytic activity of macrophages.

MATERIAL AND METHODOLOGY

Plant Material

Bengle rhizomes (*Z. cassumunar*) were purchased from the Beringharjo Market, Yogyakarta, Indonesia. Before use, these materials had been identified in the biology laboratory, University of Ahmad Dahlan, with an identification number 036/Lab.Bio/B/IV/2018.

Material

Ethanol 96% (E-Merck, pro-analysis quality), Etanol 70% (E-Merck, pro-analysis quality), n-Hexane (E-Merck, pro-analysis quality), Dichloromethane (E-Merck, pro-analysis quality), Chloroform (E-Merck, pro-analysis quality). TLC plate with silica Gel GF_{254} (Merck), Silica powder for Column (Merck), NMR JNM-ECX500R (JEOL ltd) and Complete medium (MACS® media)

Procedure

Animal Preparation

The animal care procedure in this study has been approved by the research ethics committee of the University of Ahmad Dahlan, with approval number 011804063. The test animals were male BALB mice aged eight weeks old.

Extraction and Fractionation

The rhizomes of *Z. cassumunar* were washed with water, then dried in an oven at 50 °C. The produced *simplisia* (i.e., natural ingredients used as a medicine that have not been exposed to any processing and, unless stated otherwise, are in the form of dried materials) were ground into a powder and subsequently macerated using 96% ethanol at a ratio of 1:4. Afterward, the produced macerate was evaporated in a rotary evaporator to obtain a thick extract. Fractionation was carried out by successive fractionation. Thirty grams of the ethanol extract of *Z. cassumunar* were fractionated with n-hexane until no more compounds were dissolved in n-hexane. Then, the non-soluble part was fractionated with chloroform. The fractions obtained were further examined by thin-layer chromatography (TLC).

Macrophage Isolation

After fasted for 12 hours, the test mice were anesthetized using chloroform. The abdominal skin was cut open, and the peritoneal was cleaned using 70% ethanol. Ten ml of complete medium was injected into the peritoneal cavity, which was then massaged for 3 – 5 minutes. The cavity was pressed by two fingers to remove the peritoneal fluid; this fluid was aspirated using a syringe injection (by selecting parts that had no fat and far from the intestine). The number of cells were counted and added with complete medium to adjust the number of macrophages to 2.5 x 10^6 mL^{-1} cells.

Testing of Phagocytic Activity

Cells in 200 μL suspension were grown in each well and incubated using an incubator that was set at 5% CO_2 and a temperature of 37°C for 24 hours. The medium was removed and added with 50 μL latex and, then incubated using an incubator at 5% CO_2 and a temperature of 37 ° C for 1 hour. The suspension was removed and fixed with methanol for 3 minutes, then the methanol was removed. This mixture was colored with 10% Giemsa for 30 minutes and the coverslips were washed in distilled water. Finally, the cells were viewed under a microscope with 400x magnification (Nurkhasanah, Santoso, and Fauziah 2017).

Column Chromatography

Column chromatography was performed using silica powder with 230 – 400 mesh particle size in combination with the elute of n-hexane: dichloromethane (1:1). One gram of the chloroform fraction was ground with 5 grams of silica powder and stored at the top of the silica column. This process involved gradient elution for the mobile phase (i.e., a mixture of n-hexane: dichloromethane) and was examined using thin-layer chromatography.

Identification

The subfraction that showed the best phagocytic activity of macrophages was then prepared for 1H-NMR and 13C-NMR analyses. This procedure used a JNM-ECX500R spectrometer operated at 500MHz Superconducting Magnet.

Analysis

The macrophage activity was observed from the cells exhibiting active phagocytosis and the phagocytic index. Active macrophage cells are the percentage (x100%) of the number of latex cells among the total macrophages in the field of view **(Chairul, 2009)**, while the index is the percentage of a total number of macrophages that phagocyted latex particles among the total macrophages in the field of view **(Hartini, Wahyuono, and Widyarini, 2013)**.

$$\text{Active phagocytic cells (\%)} = \frac{Number\ of\ active\ macrophage\ cells}{total\ of\ macophage\ cell} \text{ x } 100\%$$

$$\text{Index of phagocytosis} = \frac{total\ antigen\ phagocyted}{total\ of\ active\ macophage\ cell}$$

Statistical analysis

The results were analysed statistically using Normality, Homogeneity, ANOVA, and LSD (Least Significant Differences); all of which were processed in SPSS version 22. The normality test result is accepted if the *p*-value is >0.05, which means that the data are normally distributed. The homogeneity test result is accepted if the *p-value* is >0.05, which represents homogeneously distributed data. The ANOVA result is accepted if the *p*-value is <0.05, which signifies a significant difference in the treatment group. Meanwhile, the LSD test is a test performed as a reference to determine whether or not the averages of two treatments are statistically different.

RESULTS AND DISCUSSION

An immunomodulator is a drug or a pharmacological agent that regulates the immune system. The ethanol extract of *Z. cassumunar* has been reported to exhibit immunomodulatory activity by increasing the secretion of ROI. ROI secretion shows the increased activity of macrophage cells. Macrophages with their phagocytosis mechanism are the main phagocytic cells that ward off or deflect pathogens **(Abbas, Litchman and Pillai, 2012; Akrom, 2013; Baratawidjaja and Rengganis, 2014; Hartini et al., 2013; Nurkhasanah et al., 2017)**.

Based on Figure 2, Figure 3, Table 1 and Table 2, the

Figure 1 *Z.cassumunar* rhizome.

Table 1 RF values and colors of the spots on TLC.

Spot	RF	Curcumin		n-Hexan		Ekstrak		Kloroform	
		UV 254	UV 366	UV 254	UV 366	UV 254	UV 366	UV 254	UV 366
1	0.027	Orange	Orange	-	-	-	-		-
2	0.088	Orange	Orange	-	-	-	-		-
3	0.150	-	-	-	-	Blue	-	Blue	-
4	0.244	Orange	Orange	-	-	Orange	Orange	Orange	Orange
5	0.522	-	-	Blue	-	Blue	-	Blue	-
6	0.577	-	-	Blue	-	Blue	-	Blue	-
7	0.844	-	-	Blue	-	Blue	-	Blue	-

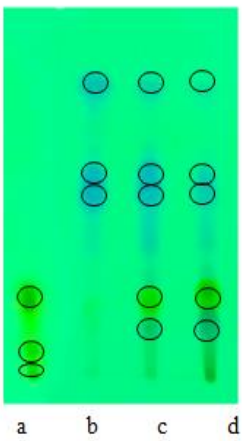

Figure 2 Profile TLC using the mobile phase chloroform: dichloromethane and stationary phase silica gel F254: a. curcumin b. n-hexan fraction c. bengle extract d. chloroform fraction.

Z. cassumunar extract, hexane fraction, and chloroform fraction significantly increased the active phagocytic cells and phagocytic index (p <0.05). Of the five test results, the highest increase in phagocytic activity was exhibited by the chloroform fraction, as indicated by active phagocytic cells (SFA) = 62.090 ±6.947 and phagocytic index (IF) = 1.638 ±0.079. Meanwhile, the lowest increase was shown by the hexane fraction, with SFA = 27.923 ±2.941 and IF = 1.476 ±0.227. These results are influenced by differences in the compounds of the fractions and *Z. cassumunar* extract. For instance, the hexane fraction only produced three spots with RF values = 0.522, 0.577, and 0.844 during the TLC procedure. Meanwhile, the extract and the chloroform fraction each had five spots with RF values= 0.15, 0.244, 0.522, 0.577, and 0.844.

The increase in phagocytic activity can occur in two ways, namely by the process of oxidative and non-oxidative. Oxidative processes increase the use of oxygen, myeloperoxidase, hydrogen peroxide, and hexose monophosphate that destroy bacteria. Hydrogen peroxide, superoxide anion, and nitric oxide secreted within the phagolysosome generate toxic oxygen metabolites that can be used to kill bacteria. Non-oxidative processes occur due to the influence of various proteins, such as hydrolytic enzymes, cationic proteins, lysozyme, lactoferrin, and nitric oxide synthase (NOS). Nitric oxide synthase can increase NO production from macrophages in the spleen with the help of IFN-γ and TNF-α **(Bermudez and Young, 1989; Ferrari, 2011; Ishimoto et al., 2008)**.

Z. cassumunar is a Zingiberaceae family that has curcumin. Curcumin is known to give antioxidant and immunostimulant effects by increasing T-cell proliferation, natural killer (NK) cell activity, and the activity of NO. One compound marker of *Z. cassumunar* is phenylbutanoid, which contains a phenyl group that initiates antioxidant effects and can support the immune system. Anti-oxidants are often associated with the immunostimulatory potential **(Ak, 2008; Chairul, 2009; Jayaprakasha, Jaganmohan, and Sakariah, 2006; Lu et al., 2008; Taechowisan, Suttichokthakorn, and Phutdhawong, 2018; Yadav et al., 2005)**.

Table 2 The phagocytic activity of the extract and fractions of *Zingiber cassumunar*.

Group	Active Phagocytic Cells (%)	Phagocytic Index
Control	8.731 ±4.080	1.641 ±0.387
n-Hexane Fraction	27.923 ±2.941*	1.476 ±0.227
Chloroform Fraction	62.090 ±6.947*	1.638 ±0.079
Z.cassumunar Extract	39.194 ±1.597*	1.215 ±0.105*

Note: *Showing a significant difference (p <0.05).

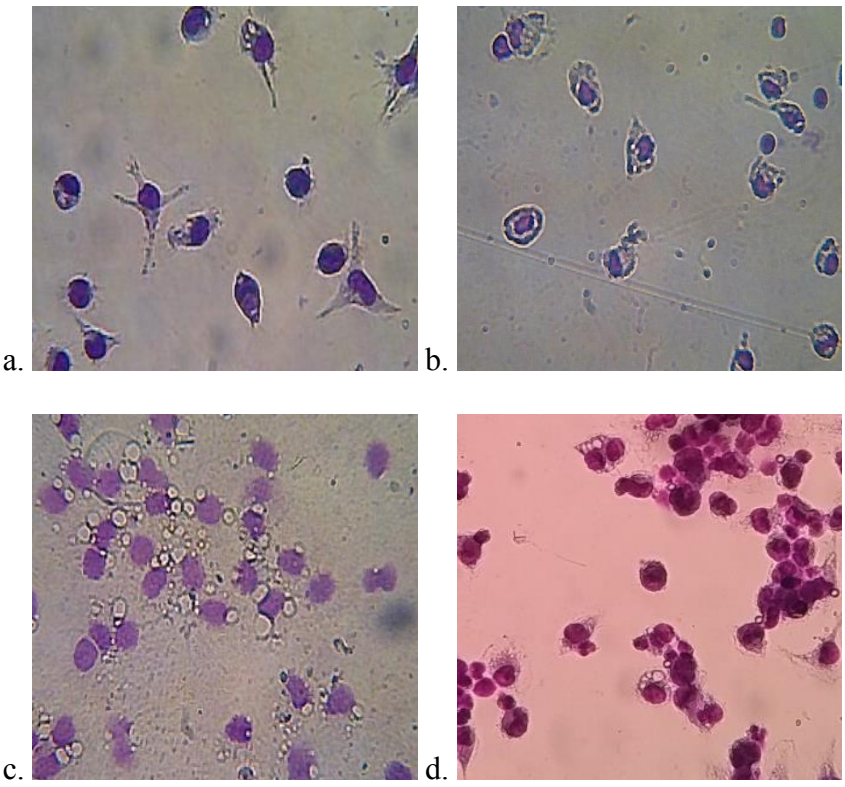

Figure 3 The Phagocytic activity of macrophage cells treated with: a. Normal group, b. Fraction of n-hexane, c. Fraction of Chloroform, d. Bengle extract.

The chloroform fraction was separated using column chromatography, and it produced five subfractions, as shown in Figure 4. These subfractions were then tested for their phagocytic activities, and the results are presented in Table 3. Table 3 shows that F1, F2, F2C, F3, and F4 influenced the phagocytic activity of macrophages. The highest activity was exhibited by subfraction F3 with SFA= 64.333 ±1.780 and IF= 1.598 ±0.059, followed by F4, with SFA= 44.943 ±2.944 and IF= 1.702 ±0.043. Meanwhile F1, F2 and F2C exhibited poor phagocytic activities, as evidenced by their SFA (18.860 ±3.191, 27.077 ±4.482, and 15.749 ±3.026, respectively) and IF (1.608 ±0.19,0 1.646 ±0.113, 1.557 ±0.167.

Table 4 shows that F3 is an active compound that is responsible for the immunomodulatory activity, which increased the activity of the murine macrophage cells. Increased macrophage activity is influenced by the production of NO, the ROI of intra phagosome killer, phagosome acidification, the fusion of phagosomes, and Fe supply reduction. The activation of macrophages can increase phagocytosis due to the increased expression of the gene transcription. The gene of macrophages has specific functions that the same cells cannot perform while in the rest position. Phagocytosis is affected by ROS. Through the ROI, ROS kills antigens that enter macrophages. Superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen belong to the group of ROS. ROS is a highly reactive species that can kill bacteria and destroy the cells. Nitric Oxide Synthase molecules bind the cofactor tetrahydrobiopterin, then this NOS-cofactors bond produces NO, which is highly toxic to bacteria. This pathway is activated by INF-γ and is triggered by the presence of TNF-α, which increases the production of NO in the spleen **(Abbas et al., 2012; Baratawidjaja and Rengganis, 2014; Ismail, Stevenson, and Walker, 2006; Parslow et al., 2001; Tzianabos, 2000)**.

IFN-γ is a potential cytokine macrophage activator. IFN-γ will increase phagocytosis and stimulate the expressions of MHC-I and MHC-II and costimulator APC. IFN work on B cells in the transfer of IgG subclasses that enable FcyR in phagocytes and activate complement. IL-10 inhibits macrophage activity and controls non-specific immune responses and cellular immunity. The main function of IL-10 is to inhibit the production of several cytokines (TNF, IL-1, chemokines, and IL-12) and the function of macrophages in helping the activation of T cells **(Brummer, Hanson, and Stevens, 1988; Lo et al., 2019; Ravindran et al., 2019; Salim, Sershen, and May 2016)**.

Table 3 The phagocytic activity of the extract and fractions of *Zingiber cassumunar*.

Group	Active Phagocytic Cell (%)	phagocytosis Index
F1	18.860 ±3.191	1.608 ±0.190
F2	27.077 ±4.482	1.646 ±0.113
F2c	15.749 ±3,026	1.557 ±0.167
F3	64.333 ±1.780*	1.598 ±0.059
F4	44.943 ±2.944	1.702 ±0.043

Note: *Shows the best result (p <0.05).

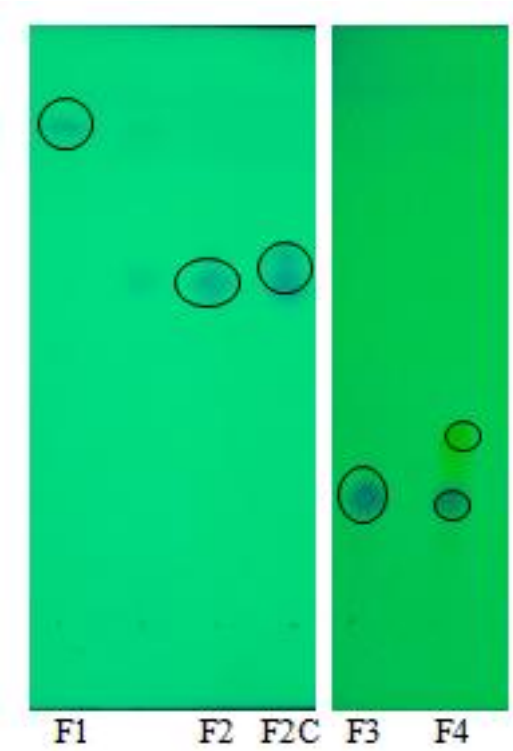

Figure 4 The TLC profile using chloroform: diklоromethan as the mobile phase and silica gel F254 as the stationary phase.

Table 4 The comparison of the NMR peaks of the active compound with other studies.

Position	^{13}C-NMR (Kuroyanagi et al., 1980)	^{13}C-NMR Result	^{1}H-NMR (Kuroyanagi et al., 1980)	^{1}H-NMR Result
1	62.1	62.3	CH_2-OH 3.68 (t,J= 6) - 2.73 (s)	CH_2-OH 3.73 (t, J=6) - 2.01 (s)
2	36.4	36.5	CH_2 2.42 (q, J= 6)	CH_2 2.44 (q,J= 6)
3	124.4	124.5	CH 5.93 (d, J=16; 6)	CH 6.06 (d, J= 16; 7)
4	132.4	132.6	CH 6.35 (d, J=16)	CH 6.39 (d 16)
1’	130.5	130.6	-	-
2’	108.8	108.7	Arom. H 6.71 (d, J=1,5)	Arom. H 6.85 (d, J=2)
3’	149.1	149.2	-	-
4’	148.6	148.7	-	-
5’	111.3	111.3	Arom. H 6.64 (d, J=8)	Arom. H 6.77 (d, J=8)
6’	119.1	119.3	Arom. H 6.87 (dd, J= 8; 1,5)	Arom. H 6.87 (dd, J= 8; 2)
7’	55.9	56.1	OMe 3.79 (s)	OMe 3.85 (s)
8’	55.8	56.0	OMe 3.80 (s)	OMe 3.87 (s)

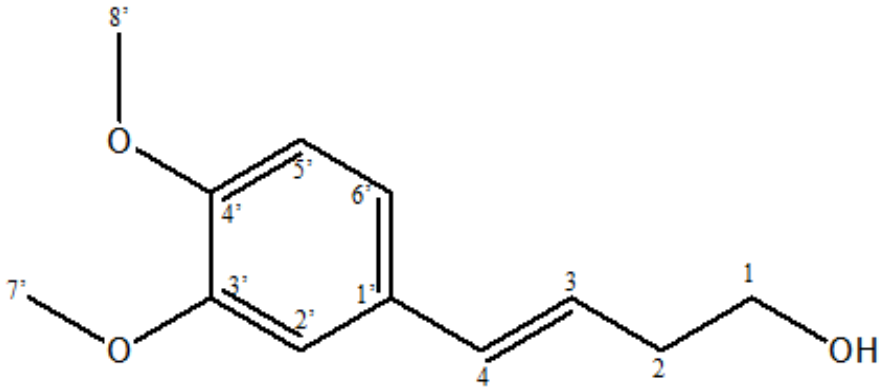

Figure 5 [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol].

TNF is the main mediator in response to a variety of antigens that cause infections. Macrophages are a major source of TNF. TNF has a biological effect that activates neutrophils, macrophages, and monocytes to eliminate the antigen and then trigger vascular cell adhesion molecule, stimulate macrophages to secrete chemokines, induce chemotaxis, leukocyte deposition, and the apoptosis of the inflammatory cells. IL-12 is a major mediator of the early nonspecific immunity against intracellular antigens. The main source of IL-12 is mononuclear phagocytes and activated dendritic cells. The biological effects of IL-12 cause the NK cells and T cells to secrete IFN **(Cavalcanti et al., 2012; Engwerda et al., 2002; Ismail et al., 2006; Salim et al., 2016)**.

The compound [(*E*)-4-(3',4’-dimethoxyphenyl)but-3-en-1-ol] is a form of light green oil with the molecular formula C12H16O3 and C6-C4 carbon framework. Table 4 shows two methoxy groups (-OCH3) at δ 3.85 (s) and 3.87 (s), which were strengthened by the presence of carbon spectra at δ 55.9 and 55.8 ppm. Moreover, aromatic carbon with ortho and meta couplings appeared as three benzene substitutions at δ 6.87 (d, J = 8, 2), 6.77 (d, J = 8) and 6.85 (d, J = 2) and was amplified at the carbon spectra δ 108.8, 111.3, and 119.1 ppm. The protons in H-7 and H-8 showed a high constant coupling of 16, which indicates a trance configuration.

Chairul (2009) suggest that the compound isolated from the identified phenylbutanoid group [(*E*)-4-(3’,4’-dimethoxyphenyl)but-3-en-1-ol] induces a more significant increase in phagocytic activity in in vivo experiments on Swiss Webster male mice than [(*E*)-4-(2', 4',5'-tri-methoxyphenyl)but-3-en-1-ol] and [(*E*)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol].

CONCLUSION

Bengle (*Zingiber cassumunar*) has the potential as immunomodulators that significantly increase phagocytic activity. The chloroform fraction has been proven to exhibit the highest phagocytic effects. Meanwhile, the subfraction that has the most substantial phagocytic effect is sub-fraction 3, which is identified as [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol].

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AN ANALYSIS OF THE USE OF MARKETING 4.0 PRINCIPLES FOR MANAGING CUSTOMERS RELATIONSHIPS IN MICROBREWERIES IN THE CAPITAL CITY OF PRAGUE

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ABSTRACT
Technological progress also affects the development of marketing, currently known as Marketing 4.0, which is characterized by digitization and indicates a connection with Industry 4.0. With digitization, companies can use a variety of tools and methods to collect useful customer data and then use it. Marketing 4.0 is mainly characterized by the fact that the customer wants to be part of the product development and wants to share her information and opinions and experience on the product. Thanks to the Internet, customers can get all the information from other customers. The aim of this analysis was to verify that microbreweries in Prague use Marketing 4.0, use digitization and adapt to the latest trends in marketing and define what methods they use to manage customer relations. The analysis is suitable for long-term monitoring of the development of this issue. Corresponding conclusions and recommendations for the future were drawn from the research findings.

Keywords: CRM; Marketing 4.0; Microbreweries; Czech Republic; Prague

INTRODUCTION
The trend today is digitalization, which has reached both marketing and other fields. In connection with the fourth technological revolution, i.e. Industry 4.0, Marketing 4.0 was created. Thanks to digitization, many new activities and procedures have been introduced into marketing, mainly thanks to the development of the Internet and social networks, which gave the customer access to information and opinions of other customers. Customers want to be part of the process and share their experiences. **(Jara et al., 2012)**. The number of microbreweries in the Czech Republic is growing every year. At the end of the 19th century, 800 to 1,000 breweries brewed beer in Bohemia and Moravia. A study on microbreweries and tourism marketing was published in 2017, where the author **Dunn and Kregor (2017)** states that the new era of marketing, i.e. the marketing era 4.0, introduced in 2017, was also reflected in the brewing industry. It is mainly thanks to social networks that made microbrewery's communication with customers is important, and this caused strengthening loyalty, sharing information and engaging customers in the brewing process. Moreover, microbreweries should also focus on different alternatives to delivering beer to customers and address the specific needs of their customers **(Dunn and Kregor, 2017)**. In the area of Prague there are 46 microbreweries that are located throughout the city. Some of these microbreweries are in the very center of Prague near Wenceslas Square, such as the Two Cats Brewery, Na Národni Brewery, or U Fleků. Other breweries are located on the outskirts of Prague, such as the Počernický Brewery. Most of these microbreweries have an on-site restaurant where meals are served together with their beer. One of the microbreweries, the Lajka microbrewery, is also unusually connected with coffee. Each of these microbreweries brews its original beer with its original name and original ingredients. As Prague is a frequent tourist destination, microbreweries also use a variety of ways to attract tourists. This especially applies to microbreweries in such destinations where there are many tourists. These tourists are attracted by microbreweries for originally crafted beer, various tastes, and mainly the possibility to buy beer in a PET bottle, in a glass bottle, buy a souvenir, or attend interesting events.

The trend of microbreweries in the Czech Republic is still on hold. In 2018, there were already 451 microbreweries in the Czech Republic and according to current trends the number will increase **(Stratilík, 2018)**. In 2015, there were 330 microbreweries in the Czech Republic experiencing a boom in the field. The top level of the Czech beer boom is out of sight and beer producers themselves claim that they do not compete with each other, and as there is a high demand for beers from microbreweries on the market, these microbreweries can still exist. There is a rather friendly atmosphere among the breweries, and they can also help each other. The only thing that can stop this boom in the field of microbreweries is the lack of hops, which is ordered more than a year in advance **(Ekonom, 2015)**. Compared to Europe, the number of microbreweries is the highest in the

UK, with up to 2378 microbreweries in 2018. The Czech Republic holds the top ten in the number of microbreweries **(Brewersofeurope, 2019)**. There are also large industrial breweries in the Czech Republic, including Plžeňský Prazdroj, Budějovický Budvar, Staropramen, and Zlatopramen **(Pivní.info, 2019)**. Beer as such is one of the most consumed alcoholic beverages in the Czech Republic. 141 litres of beer per citizen were consumed in 2018 **(Kolářová and Kolářová, 2019)**.

To support customer relationship management, microbreweries need to realize the importance of increasing customer loyalty and strengthening their business image. Today, unpredictable customer trends may occur. It is necessary to respond to these trends to keep the company interesting for its customers. This is also crucial for microbrewery businesses **(Porter and Donthu, 2008)**.

Marketing 4.0

The technological development of digital media is a historical necessity. These developments have affected cultural, educational, social and political dimensions, which also affect digital media **(Priyowidodo et al., 2019)**. Developments in technology that have occurred in recent decades have greatly changed the world and also the marketing industry. Thanks to this development, you can be labeled as participants in the age of Marketing 4.0. Today's customers require something EXTRA; they want more to satisfy their needs. Customers are looking for values that are defined by Marketing 3.0 and want to be part of marketing and to be directly integrated with the product. These customers use information technology to share their experience and that is why marketing is not focused on the product but the customers **(Łukowski, 2017)**. Marketing has evolved from Marketing 1.0 to 4.0. Marketing1.0 was focused on the product and on increasing its sales only **(Łukowski, 2017)**. These products were manufactured in rather small numbers and were intended for a large group of customers. The best example is the Ford T strategy designed by Henry Ford **(Fuciu and Dumitrescu, 2018)**. Marketing 2.0 already addressed the needs of customers. There were more products on the market and the customer could choose from and be getting well informed about the products **(Łukowski, 2017)**. Marketing 4.0 was based on technology development and can be defined as meeting the needs and wishes of customers **(Fuciu and Dumitrescu, 2018)**. **Kotler, Kartajaya and Setiawan (2017)** described Marketing 2.0 as being focused on customer and marketing. A turning point occurred in Marketing 3.0, when customers were already people with specific feelings and desires **(Kotler, Kartajaya and Setiawan, 2017)**. Emotions and a kind of emotional marketing were also included in this era of marketing. This was to place consumers on the level of thinking and sentient beings that have certain wishes and values **(Kotler, Kartajaya and Setiawan, 2010)**. Thus, the term Marketing 4.0 is related to the fourth industrial revolution. You can also see this designation in Industry 4.0 **(Drath, Horch, 2014)**. This Marketing 4.0 was introduced in 2017 and according to **Kotler, Kartajaya and Setiawan (2017)**, it combines offline and online marketing. It is a combination of existing marketing and digital elements. These digital elements represent social networks, for example, which can be used as a tool for communication with customers, but also for evaluating and sharing customer opinions **(Nowacki, 2014)**.

Marketing of the new era is also reflected in the marketing of breweries. In the study published by **Dunn and Kregor (2017)** stated that in order to support and develop the brewery, there must be support breweries via social networks, having different alternatives of supplying beer to its consumers, and address the needs of their visitors to secure their reputation and build awareness of the brand in tourism **(Dunn and Kregor, 2017)**.

Customer Relationship Management

There are many definitions in the literature and scientific articles of what Customer Relationship Management means. Customer Relationship Management can also be defined as a method or strategy to increase customer loyalty through communication and information gathering by the company **(Swift 2000)**. Customer Relationship Management is an effective marketing strategy that is a key process that is needed to sustain growth and maintain existing and attract potential customers **(Ližbetinová et al., 2019)**. According to **Parvatiyar and Sheth (2002)**, Customer Relationship Management is considered to be a strategy. This strategy targets particular potential customers that the company wants to acquire. This strategy works only with the support of marketing, customer center and sales. The aim is to bring more added values to the customer.

Payne and Frow (2005) point out that Customer Relationship Management is seen as a direct mail rather than a targeted customer loyalty strategy. With this view, the correct use of Customer Relationship Management may be compromised, as managers often think of loyalty programs or the database created in the Call Center. Thanks to digitization and progress, it is also important to keep in mind the social networks, which today play an important role in terms of customer loyalty **(Holsing and Olbrich, 2012)**.

Microbreweries

The brewery can be distinguished from one another according to the amount of beer that the brewery brews. Breweries can be divided into five categories, which vary according to the annual beer production. Domestic breweries have up to 10 litres of beer per batch.

Table 1 Number of microbreweries in Europen countries **(Brewersofeurope, 2018)**.

Country	No. of microbreweries	Country	No. of microfreweries
Great Britain	2,378	Spain	502
France	1,000	Sweden	332
Germany	824	Denmark	157
Switzerland	818	Austria	129
Italy	693	Portugal	115

These amateur beer enthusiasts are very important in microbreweries, since many of these amateurs may eventually become founders of microbreweries. Other breweries are also microbreweries that have an annual beer production of up to 10,000 hl, a brewery with a restaurant of up to 200,000 hl, regional breweries of up to 500,000 hl and industrial breweries of over 500,000 hl of annual beer production **(Kozák, 2017)**.

The number of microbreweries in the Czech Republic has varied over the years. At the end of August 2018, there were 451 microbreweries **(Stratilík, 2018)**. In comparison with the Czech Republic, in 2017, there were microbreweries in the following European countries (Table 1). Compared to European countries, the Czech Republic was placed 7th in 2017 with 402 microbreweries **(Brewersofeurope, 2018)**.

Like other industries, brewing is developing. Most Czech mini-breweries brew bottom-fermented beer, but there are also many mini-breweries where they experiment in beer production. One of these experiments is spontaneous fermentation, where the spontaneous process requires a completely different approach from microbreweries and puts more emphasis on hygiene **(Stratilík, 2018)**. As such, brewing contributes significantly to the economy. In the EU between the years 2008 and 2013, excise duty on beer of around EUR 10 billion was levied. Compared to the US in 2014, total excise tax and VAT revenues were $ 11 billion **(Ignacio and Higgins 2016)**. In the Czech Republic, the income from an excise tax on beer was CZK 4,775,528 thousand **(State Treasury, 2019)** and in comparison, with the USA in 2014, the Czech Republic's income from excise duty on beer was CZK 4,593,399 thousand **(State Treasury, 2019)**.

Scientific hypothesis

Hypothesis H_0 – More than 80% of microbreweries in Prague use Marketing 4.0 to manage the customer relationship.

Hypothesis H_1 – Less than 80% of microbreweries in Prague use Marketing 4.0 to manage customer relationships.

In view of the fact that a small amount of specific information has been processed on the subject under consideration yet, great attention needs to be paid to the collection and processing of basic data.

Research questions:

- Do microbreweries have their restaurant?
- Do microbreweries use SEO?
- Do microbreweries use social networks?
- Do microbreweries use content marketing?
- Do microbreweries have websites?

MATERIAL AND METHODOLOGY

The article deals with the marketing of microbreweries in Prague. The basic questions dealt with whether microbreweries in Prague have their restaurant, use SEO and other analytical methods, whether they use social networks, have websites, use content marketing, customize products, sell souvenirs, provided restaurant reservations, delivery and whether they use loyalty programs. From a theoretical point of view, microbreweries should also use Marketing 4.0 as part of customer relationship management.

The data were collected using qualitative research, structured interviewing, and interviewing in person at microbreweries in Prague. Thanks to the chosen research methods, the data were obtained, analysed and statistically processed, followed by conclusions and recommendations. The main aim of this work was to find out whether microbreweries in Prague are using Marketing 4.0. for customer relationship management. The research was conducted in October 2019 in microbreweries in the city of Prague.

There are 46 microbreweries in Prague, all of which were included in the research. And this makes this sample complete. Statistical methods were used for complex research sample.

Microbreweries in Prague:

Bubeneč Praha, Cobolis Praha, Dva Kohouti Praha, Hostivař H1 + H2 Praha, Jihoměstský pivovar Praha, Kail Praha, Kbelský pivovar Praha, Klášterní pivovar Strahov, Kolčavka Praha, Ladronka Praha, Lajka Praha, Libocký pivovar Praha, Loď Pivovar Praha, Lužiny Praha, Marina Holešovice, Moucha Praha, Na Lochkově Praha, Pivovar Národní, Nedomlel Praha, Novoměstský pivovar Praha, Ossegg Praha, Pivo Karlín Praha, Pivovar Kunratice – muflon, Pivovar Uhříněves, Pivovarský dům Praha, Počerňák Praha, Počernický pivovar Praha, Řeporyjský pivovar Praha, Spojovna Praha, Suchdolský Jeník, Šedivák Praha, Třebonický rukodělný pivovar, Trilobit Praha, U Bansethů Praha, U Bulovky Richter Pub Praha, U Dvou koček Praha, U Fleků, U Medvídků, U Supa Praha, U tří růží, Victor Praha, Vinohradský pivovar Praha, Vojanův Dvůr Praha**.**

Statistical analysis

Another method we used to reject or not reject the hypothesis H_0 is Pearson's Chi-Squared test. The basic assumption was that 80% of microbreweries use 4.0 marketing to manage customer relationships, focusing on research questions and significance level $\alpha = 0.05$ and conducting a significance test. The critical value of the chi-square at a number of freedoms 4 is 9.49. The calculated chi square value is 9.21. This value is less than the critical value and therefore we do not reject our hypothesis H_0 and reject the hypothesis H_1. For statistical analysis was used Social Science Statistics, the year 2020.

RESULTS AND DISCUSSION

In a complete sample of microbreweries, data were collected concerning the restaurant itself, the use of SEO and other analytical tools, the use of content marketing, product customization, souvenir sales, distribution and the use of loyalty programs.

The first research question was whether microbreweries run a restaurant. Some microbreweries have their restaurant where they brew very well and have a microbrewery only as a supplement.

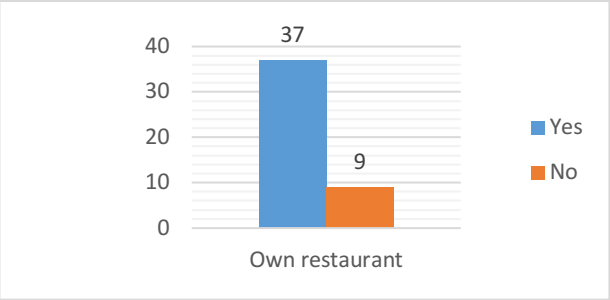

Figure 1 Own restaurant.

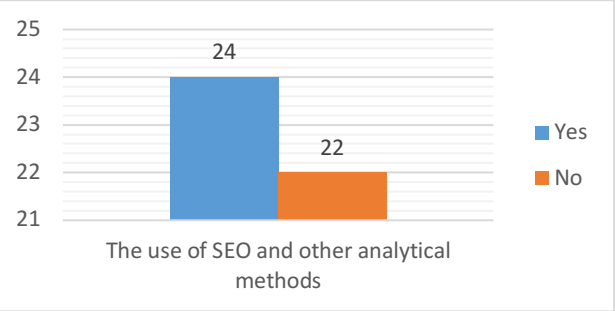

Figure 2 The use of SEO and other analytical methods.

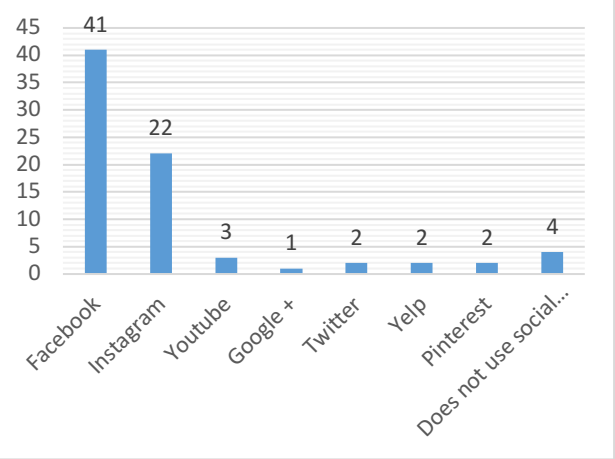

Figure 3 The use of various kinds of social networks.

Some microbreweries only brew beer and do not have a place or restaurant where they can tap their beer. Results are presented in Figure 1. 80.4% of microbreweries have their restaurant where they top their beer and only 19.6% of microbreweries do not own the restaurant or there sell their beer to other restaurants where they top their as well as types of beer.

The use of SEO sites in microbreweries is almost even. Results are presented in Figure 2. 52.1% responded positively that they use SEO on their webpages and also use other analytical methods such as Google Analytics. 47.9% of microbreweries do not use these analytical methods at all. Microbreweries should use these analytical methods to find out important information regarding their customers. SEO is a necessary system for every business owner who has a website **(Berman, Katona, 2013)**. Thanks to SEO, it is possible to improve your website and also have an overview of consumer behavior **(Yang and Ghose, 2010)**. This allows microbreweries to find out what customers are doing and looking for on the web.

Another part of the research concerned social networks, i.e. what social networks microbreweries use. The results are presented in Figure 3. Most microbreweries use social network Facebook. The second most used social network is Instagram. Four of the microbreweries surveyed do not use social networks at all. For these microbreweries, the website is also at a poor level. Some microbreweries also use a social network to share YouTube videos. Most microbreweries combine social networks Facebook and Instagram. According to **(Clark et al., 2017)** if social networks are maintained and communication is maintained, social networks are very beneficial for business. Here you can give events that happen in microbreweries but also photos and more. It is therefore important that microbreweries communicate via social networks.

As for the use of social networks it was also examined whether microbreweries use content marketing. The results are presented in Table 2. The research found that five microbreweries do not use content marketing at all. Other microbreweries as for the use of this type of marketing, hire a special person to take care of their social networks or manage to create this content that is of interest to their customers themselves. The use of content marketing was evaluated on a five-point scale, with 1 - not fully utilized and 5 – fully utilized. **Rowley (2008)** argues that content marketing is particularly important for strategy and marketing communications. Thanks to this content marketing we can use it well in communication with customers. According to research, most microbreweries use content marketing, which is good for their communication with the customer.

The statistical processing shows that the arithmetic mean is 3.217 and occurs in the range of "neither use nor uses" and "rather uses". On a scale of 1 to 5, the median is 3.

Table 2 Using content marketing on scale.

	1 - not fully used	2 - rather not used	3 - neither uses nor uses	4 - rather uses	5 - fully utilized
Content marketing	5	8	15	8	10

Table 3 Microbrewery websites rated on scale.

	1 - completely outmoded	2 - rather outmoded	3 -neither modern nor modern	4 - rather modern	5 - completely modern
Website	5	8	10	13	10

The standard deviation is 1.478 and indicates that most microbreweries do not deviate from the average 1.478. Another part of the research dealt with the website. All microbreweries surveyed have websites. The results are presented in Table 3.

The research analysed web pages of microbreweries in terms of modernity and suitability for customers. The website was ranked on a five-point scale, with 1 – completely non-modern and 5 – completely modern. For microbreweries that lively communicate on social networks, the website was "very" or "completely" modern. For microbreweries that do not communicate so often on social networks, or their posts are not of interest to users, the website is more neutral or out of fashion.

The statistical processing shows that the arithmetic mean is 3.326 and occurs in the range of "neither modern nor modern" and "rather modern". On a scale of 1 to 5, the median is 4. The standard deviation is 1.857 and indicates that most microbreweries do not deviate from the average by 1.857. 21.8% of microbreweries on the scale were rated as "completely modern" and "neutral". Most microbreweries, namely 28.2% of microbreweries, were rated as rather modern. All websites provide contacts, opening hours on where the microbrewery can be found, and in many cases, a menu is also provided.

The graph shows the evaluation of the other questions examined.

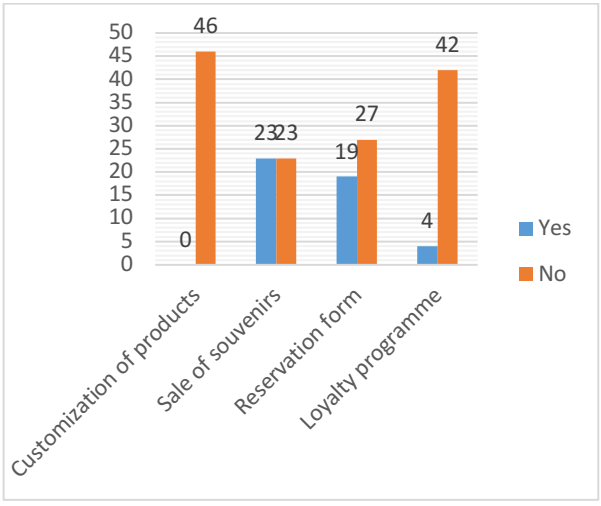

Figure 4 The evaluation of the remaining questions.

In the last part of the inquiry, the microbreweries were asked whether they customize products, sell souvenirs, whether they have a reservation form for a restaurant and whether they offer a loyalty program. The results are presented in Figure 4. None of the microbreweries responded positively to the customization of their products. The brewing process depends on the demand and the brewer, but microbreweries do not include services such as customized labels or beer on the client's request. 50% of microbreweries sell their souvenirs. The most interesting souvenir is a cycling jersey of the microbrewery Hostivař H1, which is sold in the style of beer with the logo of the microbrewery. The most frequented souvenirs are glasses with the brewery logo, as well as trays, pencils, or baseball caps. Restaurants having a microbrewery as a supplement to the restaurant do not usually have such souvenirs; people can only buy their beers bottled into PET bottles. Souvenirs in the travel industry have a strong symbolic value associated with pleasant travel experiences and memories **(Baizerman et al., 1994)**. As beer tourists can buy a souvenir, they can also take away something that reminds them of a microbrewery. According to **(Collins-Kreiner and Zins, 2011)**, souvenirs can affect the tourist experience and enhance the emotion that can affect the tourist.

A reservation form is a form that can be used to make a restaurant reservation on a website without having to call the restaurant just by filling in the name, time, contact and number of people and the microbreweries arrange a table for the hour. If the restaurant is already full, they call the contact person to inform that the reservation is no longer possible to be made. None of the microbreweries have an interactive restaurant map where the customer could choose the specific place they wish to have. Only four microbreweries use the loyalty program. The loyalty programs for all these microbreweries work on the principle of points. They earn points for the beer they drink, and they can either buy a souvenir or beer for those points. To support customer relationship management, microbreweries need to realize the importance of increasing customer loyalty and enhancing its business image. Today, unpredictable customer trends can occur. It is necessary to respond to these trends to keep the company interesting for its customers. This is also crucial for microbrewery businesses **(Porter and Donthu, 2008)**. Microbreweries in Prague use various possibilities to increase customer loyalty. In particular, the sale of souvenirs. These loyalty programs have only 4 microbreweries. Youjae Yi and Hoseong Jeon report in the Journal of the Academy of Marketing Science that loyalty programs are important for perceived value and brand loyalty **(Yi and Jeon, 2003)**. According to **(Zakaria et al., 2014)** is an important relationship between loyalty program and customer satisfaction, loyalty, and customer satisfaction, and they also work against the customer's transition to competition. As already mentioned, microbreweries do not prefer these loyalty programs yet.

The trend of microbreweries is still growing and as **Stratilík et al. (2018)** said, microbreweries will continue to grow. Because the microbreweries can feed themselves and there is a demand for the beer that is brewed in microbreweries, this boom has no end. The only thing that can stop microbrewery is the lack of hops, which is ordered more than a year in advance **(Ekonom, 2015)**. There is also a boom in microbreweries in the United States; from 8 to 2768 **(Moore, Reid and McLaughlin, 2016)**. The boom of microbreweries is not limited to Europe, but also other countries. As in Britain, where the number of micro-breweries is increasing, and microbreweries gain a larger market share **(Cabras and Bamforth, 2016)**. It can be argued whether microbreweries with such growth rates compete with large breweries in the future.

When the research questions are included in the statistical processing, we get to the average use of Marketing 4.0 at 84.88%, thus not rejecting the hypothesis H_0. To not reject or reject the hypothesis H_0, it is assumed that more than 80% of microbreweries use Marketing 4.0 and $\chi 2$ chi-square statistic has been used. The critical value of the chi-square $\chi 2$ at the number of freedoms 3 is 9.21. This value does not reject our hypothesis H_0 and rejects the hypothesis H_1.

Recommendation

Today, there is a boom in food delivery applications. Some microbreweries, but a relatively small part, use the option of delivery via the Dámejidlo.cz or Uber applications, but through these applications only food from the restaurant can be ordered. Microbreweries should extend their offer to the distribution of beer products. For example, in the Damejidlo.cz application, customers can only order a beer in PET bottles, or special large sets, such as a celebration.

Several microbreweries have poor website status. In today's digital era, the microbrewery web presentation should be a calling card for a new potential customer and an existing customer, too. For these microbreweries, it would be advisable to have a professional form of the website with SEO for search engines.

Three of the microbreweries bought an application for their smartphones, where the current menu, phone contacts, and information about the microbrewery are available. In some applications, there are also updates and links to social networks. None of these microbreweries have loyalty points that could be maintained in the mobile app. The users would log in to this app and be able to monitor the status of their points which they would receive for drinking beer and could choose their reward either in the form of free beer or souvenirs directly in the application.

The microbreweries, which are in the center of tourism in Prague, offer tours of their microbreweries. However, the microbreweries that are located in parts of Prague where tourists do not travel much do not organize these tours or organize them in exceptional cases only. For local customers who drink beer from the local microbrewery, it might be interesting to look at the microbrewery, or link it to, for example, a newly brewed type of beer.

CONCLUSION

The article was focused on the application of Marketing 4.0 in microbreweries in Prague. In the introduction, literary research was conducted, which was an introduction to the issues of Marketing 4.0, microbreweries, and CRM. The research surveyed 46 microbreweries located in Prague by personal interviewing, structured interviewing, and interviewing data. These data were processed, analysed and subsequently recommendations and formulated conclusions were made.

The article set out scientific questions that were answered by microbreweries about their restaurant, the use of SEO, social networking, content marketing, and websites. Over 80% of the microbreweries have their restaurant where customers can order something to drink or eat.

Over 52% of the microbreweries use SEO and other analytical methods. Microbreweries use social networks, especially Facebook and the second most used social network is Instagram. Two of the microbreweries also use the social network Pinterest. On these social networks, microbreweries post messages containing photographs of food, events that are going to be in the microbrewery, or photographs from the microbrewery background.

All microbreweries have websites that have been rated on a five-point scale. Some microbreweries have outdated and old websites. Other microbreweries have very modern and new websites. Three microbreweries have purchased mobile applications for their customers, where they can find out the current menu and news.

When the research results are included in the statistical processing of research questions, we get to the average use of Marketing 4.0 at 84.88%, thus not rejecting the hypothesis H_0. Not to reject or reject hypothesis H_0, it is assumed that more than 80% of microbreweries use Marketing 4.0 and a chi-square statistic has been used. The critical value of the chi-square at the number of freedoms 3 is 9.21. This value rejected the H_0 hypothesis and rejects the H_1 hypothesis.

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CONSUMPTION OF FOOD IN THE EU BY THE DEGREE OF URBANIZATION: DATA VISUALIZATION AND CLUSTER ANALYSIS OF THE EU SAMPLE

Lenka Maličká

ABSTRACT

This paper examines the consumption of one of the COICOP classes – food and non-alcoholic beverages – by the degree of urbanization on the sample of EU countries in three periods – 2005, 2010, and 2015. The share of this class in total consumption of cities, towns, and suburbs and rural areas presents the second largest item of the total consumption of all structures in question. They examined the key variable creates an input to the analysis stated in the paper. First, the data visualization is realized by creating maps of scaled consumption of food and non-alcoholic beverages in cities, towns, and suburbs and rural areas in the three periods – 2005, 2010, and 2015. The spatial distribution of data shows, that higher shares of consumption of food and non-alcoholic beverages are obtained in CEE and southern countries in all structures and all periods. Considering that consumption of food and non-alcoholic beverages is negatively correlated with GDP per capita or household expenditure per capita it is possible to conclude that countries with lower levels of GDP per capita spend more on goods of daily use. Second, based on k-means clustering, cluster analysis is stated. Similarities between EU countries in the consumption of food and non-alcoholic beverages by the degree of urbanization and with respect to socio-economic conditions are investigated. Clusters are made for all three monitored periods. In 2005 and 2010 five clusters were identified, in 2015 their number has been reduced to four. Similarities between EU countries in the consumption of food and non-alcoholic beverages by the degree of urbanization change through time. The delayed effect of the financial crisis may explain observed changes. The obvious relocation of countries is evident when comparing clusters in the period 2010 and 2015. Besides it, the most stabile cluster is the cluster, which contains core EU countries.

Keywords: city; suburb; rural area; cluster analysis; COICOP

INTRODUCTION

Currently, tendencies towards the growth of cities, towards the urbanization in the European Union (EU) countries and global terms are evident. The importance of cities relies on the concentration of GDP, work places, and social and cultural infrastructure. On the sample of EU countries, the paper compares a very important part of consumption – consumption of food and non-alcoholic beverages by the degree of urbanization in EU countries. Consumption of food is an inevitable part of human consumption. In EU countries, in 2005, 2010 and 2015 it creates in average 16.1% of the total consumption of cities, 17.6% of the total consumption of towns and suburbs and 19.4% of the total consumption of rural areas (respecting the classification of consumption known as the Classification of individual consumption by purpose, abbreviated as COICOP, for more details see Table 1) **(European Commission, 2019a)**. Besides consumption related to housing, water, electricity, gas, and other fuels, it presents one of the highest items of consumption at all degrees of urbanization. Noticeably, it varies by the degree of urbanization.

Currently, the field of urbanization is widely examined by empirical evidence. **Balk et al. (2018)** mention that most of the future population growth will take place in cities. However, the movement to the cities and their attraction is discussed in an earlier paper of **Yap (1977)**. Since then, cities became a focus of research. The size and prosperity of cities are stressed by **Batty (2011)**, the "New science of cities" is presented in **Batty (2013)**.

However, the urbanization is often examined in connection with economic performance. According to **Bertinelli and Black (2004)**, urbanization and economic growth are positively linked to each other. But as **Poelhekke (2011)** stresses, the urbanization can accelerate even under the worsening economic conditions due to rural-urban migration. Similarly, **Chen et al. (2014)** conclude that expected economic benefits from accelerated urbanization are not obtained in certain cases. They identify a group of countries where the relationship between the economic growth and growth of urbanization is not statistically significant. **Jedwab, Christiaensen, and Gindelsky (2017)** focused on such terms as rural push and urban pull factors in connection with the rural-urban migration. They deliberate over the third factor influencing

the rapid urbanization, urban push factor, which covers the rapid internal urban population growth. The large body of scientific papers is dedicated to the impact of further urbanization on the environment, e.g. **Borck and Pflüger (2019)**, **Yazdi and Dariani (2019)**, **Raheem and Ogebe (2017)** or **Marlier et al. (2016)**.

The European Commission (2019b) monitors the urbanization at the global and EU levels. Expected results of urban sprawl (continuing urbanization) describe a situation, when the urban population reaches 60% by 2030 and 68.4% by 2050. The economic power of cities is broadly discussed because it is estimated that they generate 80% of all economic growth. However, the topic of urbanization in Europe has been a point of interest for a long time. **Enyedi (1990)** mentions that East-Central European countries (socialistic countries) replicated the global process of urbanization, but it was influenced by specific factors related to their history and partisan ideology. Urban sprawl in the European Union was investigated by **Patacchini et al. (2009)**. Urban population trends are discussed in **Kabisch and Haase (2011**). Growth of cities in EU countries in the period of 1990 – 2000 and 2000 – 2006 was investigated by **Haase, Kabisch and Haase (2013)**.

Consumption of food in EU countries is an object of many papers, and it is monitored by the European Commission. The convergence of consumer attitudes to food in the EU was examined in **Trail (1998)**. Since then, many research papers with research objects related to food consumption in EU countries were published (e.g. **Dudek and Koszela, 2013**; **James, Lomax and Birkin, 2019)**. European food consumption databases are under scrutiny in **Le Donne et al. (2011)**. European Food Safety Authority **(EFSA, 2019)** collects data on food-related data. Household expenditure by the consumption purpose (COICOP) is provided by Eurostat **(European Commission, 2019c)**. Thus, even though consumption of food and non-alcoholic beverages by the degree of urbanization is monitored in EU countries (e.g. in the **European Commission, 2019a)**, it is not under the systematic surveillance of empirical evidence.

The paper's motivation is driven by the evidently increasing role of urbanized structures. The paper aims to elicit, if there are any differences in food consumption in differently urbanized structures of EU countries. Correspondently, using the cluster analysis, EU countries are divided into more homogenous groups based on consumption of food by the degree of urbanization and by selected macroeconomic variables in the three periods (2005, 2010 and 2015), from which one is linked to the period immediate to a recent financial crisis.

Paper is organized in the following manner. After the part Introduction, scientific hypotheses of research are listed. Next, the chapter Material and Methodology explains the data and instrumentation used in the paper. Results and Discussion present and discuss results obtained in the provided analysis. The paper is ended by Conclusion and References.

Scientific hypotheses

Consumption of food and non-alcoholic beverages increases with the decrease in the degree of urbanization.

In cities, the share of consumption of food and non-alcoholic beverages in total consumption is lower than in other structures.

Similarities between EU countries in the consumption of food and non-alcoholic beverages by the degree of urbanization change in respect of the recent financial crisis.

The financial crisis in 2009 influenced the consumption of all structures at all degrees of urbanization. Correspondently changes contain clusters.

Table 1 Consumption shares of EU countries (in %) on the basis of COICOP classification and by the degree of urbanization.

COICOP	Cities	Towns and suburbs	Rural areas
Food and non-alcoholic beverages	16.1	17.6	19.4
Alcoholic beverages, tobacco and narcotics	2.5	2.6	2.7
Clothing and footwear	5.3	5.3	5.0
Housing, water, electricity, gas and other fuels	29.5	27.2	26.7
Furnishings, household equipment and routine household maintenance	5.0	5.3	5.3
Health	3.6	3.5	3.6
Transport	11.0	12.4	13.1
Communications	3.6	3.6	3.5
Recreation and culture	8.3	7.9	7.2
Education	1.3	1.0	0.9
Restaurants and hotels	6.0	5.4	4.9
Miscellaneous goods and services	7.7	7.9	7.6
Total	99.9	99.7	99.9

Note: Averages are computed for 2005, 2010 and 2015 employing all EU countries; light grey indicates the lowest values, dark grey indicates the highest values, unknown consumption is excluded.

MATERIAL AND METHODOLOGY

The research involved various types of data, but all of them are extracted from the Eurostat database. The three degrees of urbanization are distinguished. The most urbanized are cities. The degree of urbanization diminishes in towns and suburbs and the rest is concerning rural areas. Data related to consumption by the degree of urbanization are covered in **(European Commission, 2019a)**. Data mentioned hereinbefore are classified according to the COICOP. Here, data are collected in five-year intervals. Hence, years 2005, 2010 and 2015 are employed in research. This is mainly influenced by the incomplete database in previous years and thus the aim to minimize the occurrence of missing data in the panel. For the research purpose, the most important of them are data on the share of food and non-alcoholic beverages consumption in total consumption of all types of urbanization structures. The socio-economic conditions of EU countries are expressed employing the data on GDP, household expenditure, unemployment rate, inflation rate and country size (population). These data are covered in **(European Commission, 2019d)** and **(European Commission, 2019e)**. GDP at market prices is expressed in per capita terms. Final consumption expenditure of households is expressed in per capita terms, too. The annual average of the unemployment rate is expressed as the percentage of the active population. Inflation is based on the food price monitoring tool, expressed as Harmonized Index of Consumer Prices (HICP, 2015 = 100) for the COICOP class of Food in the last month of the respective period (2005M12, 2010M12 and 2015M12). Country size employs the variable of the total population on 1 January.

Statistical analysis

Respecting the paper goal, two main scientific instruments are used to provide the analysis of food and non-alcoholic beverages consumption in EU countries.

First, the creation of maps serves to a visualization of the differences in the spatial distribution in food consumption by the degree of urbanization in EU countries. Maps are created using the R program for statistical computing and graphics **(R Development Core Team, 2019)**. It is a free software environment. The latest R version 3.6.1., released on July 5, 2019, is employed in the research. Packages rgdal, shape and map tools serve to the map creating.

Second, cluster analysis is realized to find similarities/differences between the consumption of food and non-alcoholic beverages by the degree of urbanization in EU countries in 2005, 2010 and 2015. Cluster analysis is provided in the R version 3.6.1, packages class, cluster, pvclust, mclust, stats, graphics, maps, LLAhclust and shapefiles. In general, the method of clustering objects into groups (clusters) is based on the principle of obtaining the highest possible similarity inside the group and the lowest similarity between different groups. In this paper, the method of k-means clustering, introduced by **MacQueen (1967)**, is used. It is an iterative optimization method, which starts from an initial division of object into *k* clusters. In a k-means clustering, the number of centroids is defined in the expertise of the author. Consequently, the number of centroids, which present a real or imaginary center of cluster, reduces the number of clusters. After the definition of the number of centroids, Euclidean distances between each object and centroids are computed. The object is allocated to the nearest initial centroid. In the next step, a new centroid for each cluster is computed. In a stepwise procedure, distances between each object and each centroid are computed again. In case a certain object is closer to another centroid, as it was in the previous step, it is shifted to another cluster. Thus, objects in clusters are rearranged. The aforementioned procedure is repeated until the rearrangement of clusters stops. The k-means clustering depends on the order of the object, thus the locally optimal solution is achieved. The k-means clustering is in details processed e.g. in **Rokach and Maimon (2005)**, **Stankovičová and Vojtková (2007)** or **Řezánková, Húsek and Snášel (2009)**.

RESULTS AND DISCUSSION

Analysis based on data visualization (maps)

The expectation that in cities the share of consumption of food and non-alcoholic beverages in total consumption is lower than in other structures has been confirmed. The combination of data extracted from **(European Commission, 2019a)** and **(European Commission, 2019c)** is projected in Figure 1. It contains nine maps created for the three degrees of urbanization and three periods.

In 2005, results show that the share of consumption of food and non-alcoholic beverages on total consumption of cities is similar to towns and suburbs, but is lower than in rural areas. It is more evident in the case of Central and East European countries (CEE) of the EU and southern countries of the EU (e.g. Italy, Spain and Portugal).

In 2010, the difference between cities and lower degrees of urbanization becomes more obvious. Higher shares of consumption of food and non-alcoholic beverages are observed in towns and suburbs and rural areas. Again, as it is in a previous period, this is observable in CEE and southern countries of the EU (including France).

In 2015, the distinction between cities, towns and suburbs and rural areas is the most obvious. The given hypothesis has also been confirmed in this case when the share of consumption of food and non-alcoholic beverages in total consumption of cities is lower than in towns and suburbs, and also than in rural areas. Consumption of food and non-alcoholic beverages increases with the decrease in the degree of urbanization. The spatial distribution of consumption of food and non-alcoholic beverages intensity emulates the results reached in 2005 and 2010.

In general, results point to the current advanced socio-economic development of cities described in **Bertinelli and Black (2004)**, **Patacchini et al. (2009)**, **Kabisch and Haase (2011)**, **Haase, Kabisch and Haase (2013)** or **Balk et al. (2018)**. Supposing that the economic activity concentrated in cities leads to higher living standards of the city population, the share of COICOP items on total consumption (see Table 1) emulates the expected behavior of developed societies. In comparison with lower degrees of urbanization, they spend more money on recreation, education, hotels and restaurants and housing including fuels.

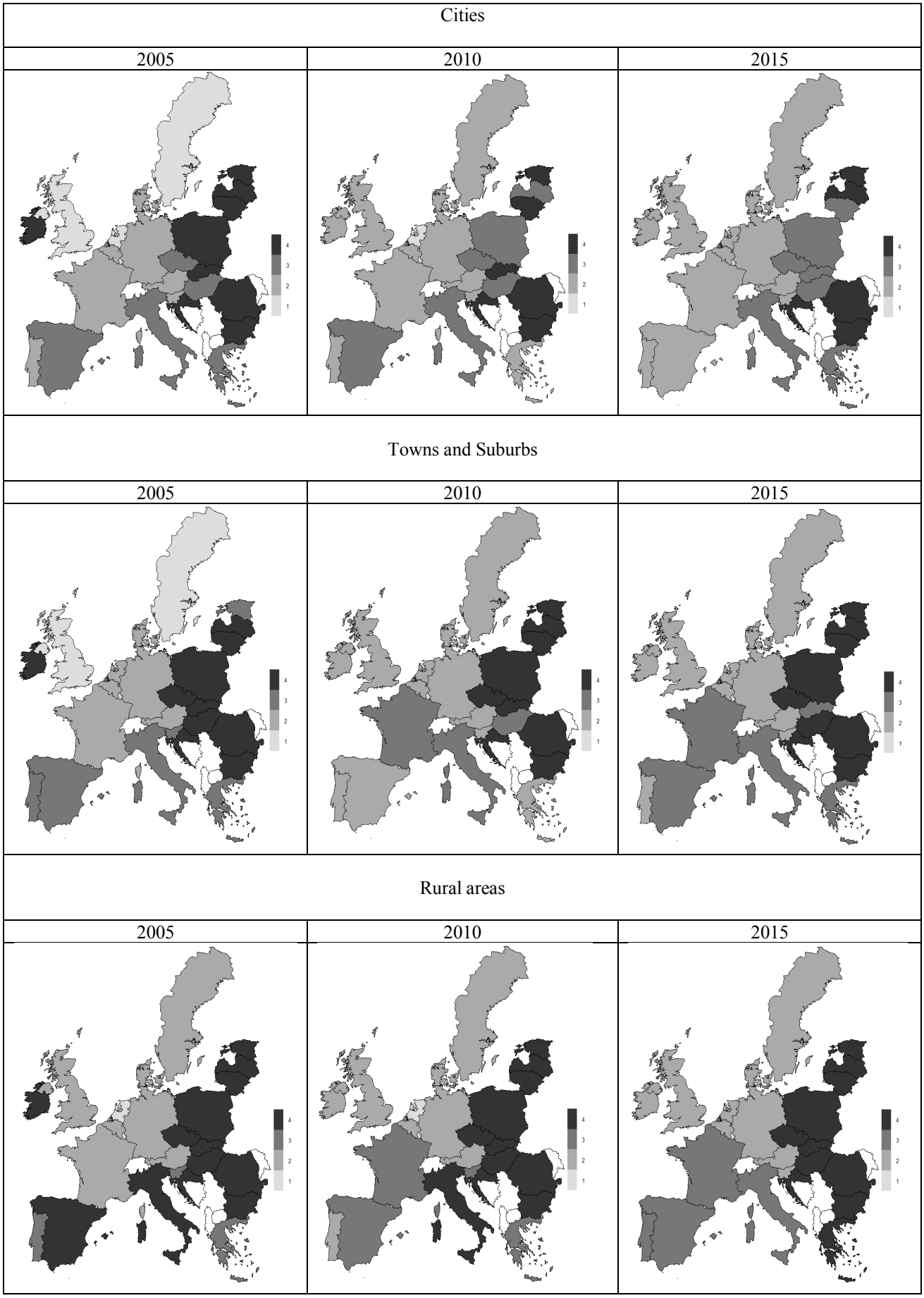

Figure 1 A comparison: Intensity of food consumption by the degree of urbanization in 2005, 2010 and 2015. Note: Scale: (1) the lightest shadow of grey refers to values lower than 0.1 (or 10%), (2) refers to 0.1 – 0.15, (3) refers to 0.15 – 0.2, (4) the darkest shadow of grey refers to values higher than 0.2.

Besides it, certain other common tendencies may be recognized. The intensity (share in total consumption) of food and non-alcoholic beverages consumption is higher in CEE countries. It may be explained by GDP, income and living standards in CEE countries, which, in fact, overcame the transition in the last decades. As mentions by **Enyedi (1990)**, CEE countries "replicated" the trend of urbanization. The urban sprawl mentioned in **Patacchini et al. (2009)** or the growth of cities, examined by **Kabisch and Haase (2011)** and **Haase, Kabisch and Haase (2013)** are present in these countries, too. However, a certain delay in the level of economic development in comparison with the core EU countries is evident. Higher values of the food and non-alcoholic beverages consumption in mentioned countries are still preferred to goods and services of no daily pattern consumption.

Cluster Analysis

Results of cluster analysis present clusters of EU countries, which are homogenous in consumption of food and non-alcoholic beverages by the degree of urbanization (cities, towns and suburbs and rural areas), GDP per capita, household expenditure per capita, unemployment rate and inflation on food. Table 2 displays the correlation coefficients of the mentioned variables.

Using the k-means clustering, EU countries are clustered into five clusters in 2005 and 2010 (see Figure 2 and Figure 3) and into four clusters in 2015 (see Figure 4).

The content of clusters in 2005 is the following:

1) Poland, Slovakia, Croatia, Romania, Latvia, Lithuania, Bulgaria.
2) Czechia, Estonia, Hungary, Malta, Slovenia.
3) Germany, France, Italy, United Kingdom.
4) Belgium, Netherlands, Sweden, Finland, Austria, Cyprus, Denmark, Luxembourg.
5) Spain, Portugal, Greece, Ireland.

The content of clusters in 2010 is the following; relocated countries are highlighted in italics:

1) Poland, Slovakia, Croatia, Romania, Latvia, Lithuania, Bulgaria, *Estonia.*
2) Spain, Portugal, Greece, Ireland.
3) Belgium, Netherlands, Sweden, Finland, Austria, Cyprus, Denmark, Luxembourg.
4) Germany, France, Italy, United Kingdom.
5) Czechia, Estonia, Hungary, Malta, Slovenia.

The content of clusters in 2015 is the following; relocated countries are highlighted in italics:

1) Belgium, Netherlands, Sweden, Finland, Austria, Denmark, Luxembourg, *Ireland.*
2) Spain, Portugal, Greece, *Slovenia, Cyprus.*
3) Poland, Slovakia, Croatia, Romania, Latvia, Lithuania, Bulgaria, *Malta, Czechia, Estonia, Hungary.*
4) Germany, France, Italy, United Kingdom.

The hypothesis that similarities between EU countries in the consumption of food and non-alcoholic beverages by the degree of urbanization change through time has been confirmed. The most significant differences are observable comparing the years 2010 and 2015. The influence of the financial crisis in 2009 may be observed in the following period of 2015 when the economic recovery brings certain rearrangements in comparison with the period before the financial crisis (2005). Correspondent changes in the content of clusters reflect the reorganization of results.

Table 2 Correlation matrix of food consumption by the degree of urbanization (cities, towns and suburbs, rural areas) and selected macroeconomic indicators.

	Cities	**Towns and suburbs**	**Rural areas**	**GDP pc**	**Household Expenditure pc**	**Unemployment rate**	**Inflation on food (HICP)**
Cities	1	0.9535	0.9504	-0.7614	-0.8415	0.2729	-0.2269
Towns and suburbs		1	0.9695	-0.7692	-0.8566	0.2548	-0.1737
Rural areas			1	-0.7549	-0.8439	0.2737	-0.2241
GDPpc				1	0.9185	-0.3657	0.2800
Household Expenditure pc					1	-0.3422	0.3166
Unemployment rate						1	0.1512
Inflation on food (HICP)							1

Note: Correlation coefficients; using the observations 1:1 – 28:3; 5% critical value (two-tailed) = 0.2146 for n = 84.

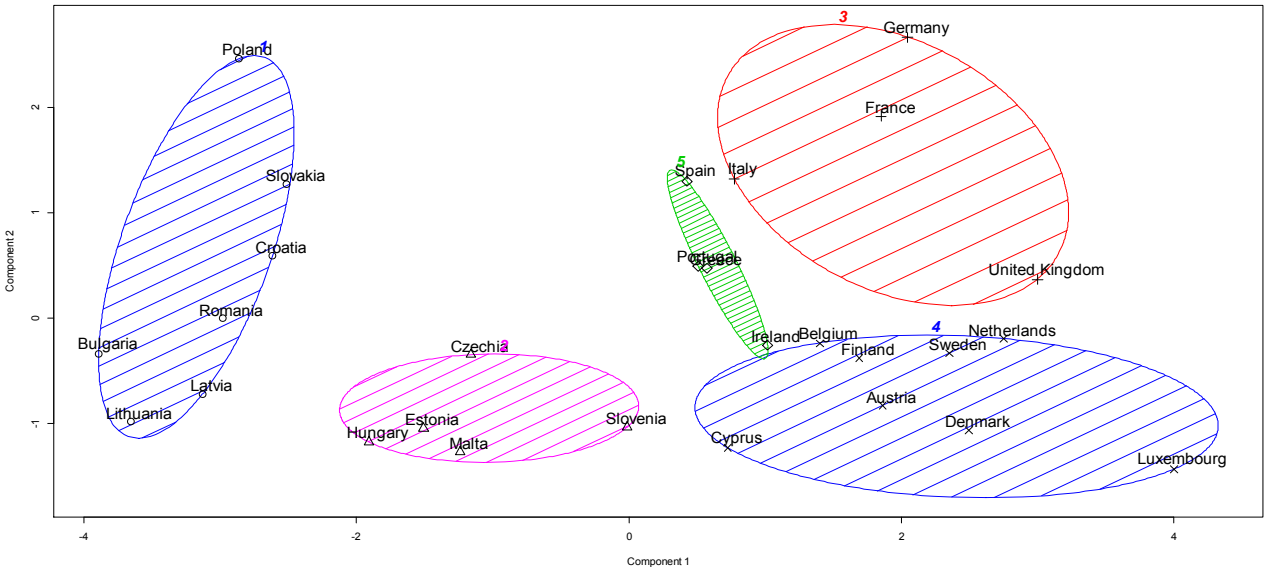

Figure 2 Clustering EU countries in 2005. Note: k-means clustering.

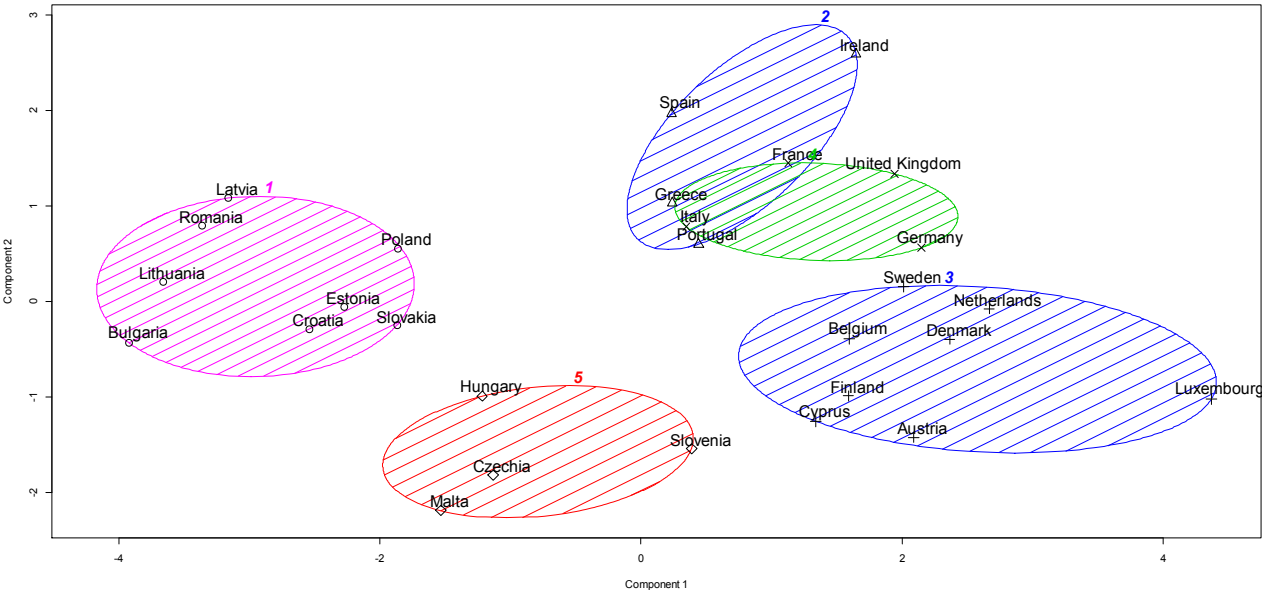

Figure 3 Clustering EU countries in 2010. Note: k-means clustering.

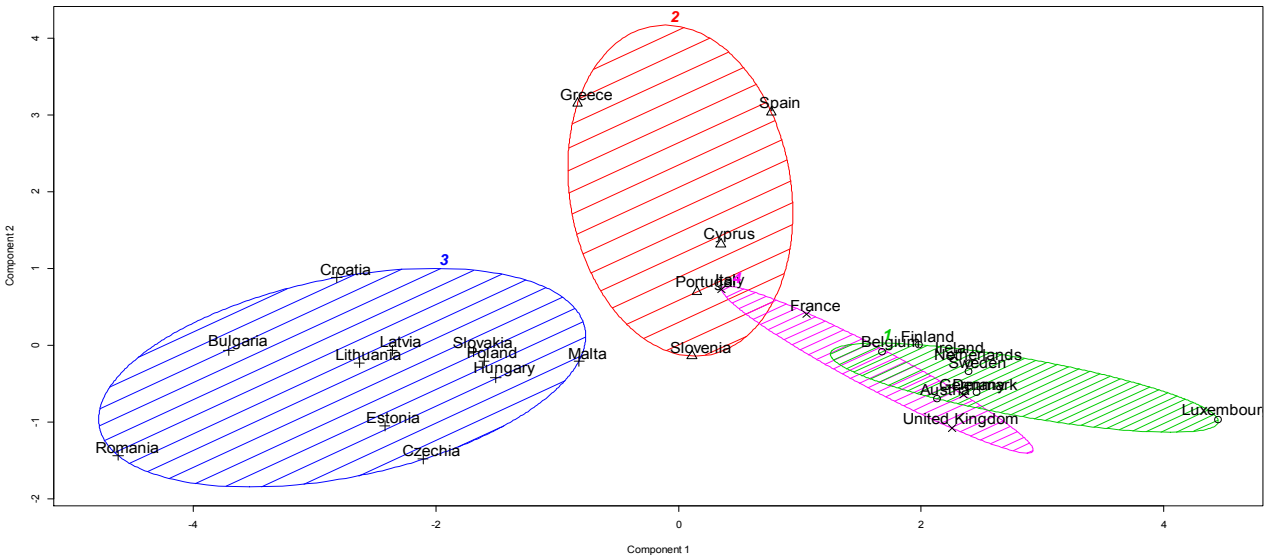

Figure 4 Clustering EU countries in 2015. Note: k-means clustering.

Comparing periods 2005 and 2010, clusters seem to be stable, without evident relocation of countries. Only Estonia moved from the cluster of Czechia, Estonia, Hungary, Malta and Slovenia (2005, cluster 2) to cluster of Poland, Slovakia, Croatia, Romania, Latvia, Lithuania and Bulgaria in 2010 (2010, cluster 1).

In 2015 obvious relocation of countries may be observable. First, contrary to periods 2005 and 2010, four clusters are created. Hence, in 2015 two clusters joined to one (2015, cluster 3). The rest of the clusters stayed almost unchanged.

The most stable cluster is cluster, which contains core EU countries (Germany, France, Italy and the United Kingdom). It remains without any changes.

Ireland moved from the cluster of Spain, Portugal, Greece and Ireland (2010, cluster 2) to cluster of Belgium, Netherlands, Sweden, Finland, Austria, Denmark and Luxembourg (2015, cluster 1).

Slovenia moved from the cluster of Czechia, Estonia, Hungary and Malta (2010, cluster 5) to cluster of Spain, Portugal and Greece (2015, cluster 2).

Cyprus moved from the cluster of Belgium, Netherlands, Sweden, Finland, Austria, Cyprus, Denmark and Luxembourg (2010, cluster 3) to the cluster of Spain, Portugal and Greece (2015, cluster 2).

Clusters presented in Figure 2, Figure 3 and Figure 4 correspond approximately to 77% of the variability of the analysed problem.

Chen et al. (2014) examined the worldwide urbanization by comparing the year 1980 and 2011. According to their results, current EU member countries did not overcome a dramatic wave of urbanization when comparing e.g. to China or Central Africa. At the base of Figure 1, it is obvious that the intensity of the consumption of food and non-alcoholic beverages changed in the cities predominantly. This is observable in 2015. The relocation of countries in the cluster analysis (see Figure 2, Figure 3 and Figure 4) is thus importantly influenced by other variables of macroeconomic nature extracted from **(European Commission, 2019d)** and **(European Commission, 2019e)**. As many of the created clusters are sensitive to changes in macroeconomic conditions, the stabile cluster of core EU countries resists external shock in the form of a financial crisis.

Research on household consumption in EU countries provided by **Dudek and Koszela (2013)** points to certain accordance with the results of the cluster analysis. These authors mention similarities between the Czech Republic and Estonia (in this research both involved in cluster 3 in 2015), Cyprus, Greece and Portugal (cluster 2 in 2015) or Poland, Hungary and Slovakia (the cluster 3 in 2015). Although the field of food consumption is not so rarely employed to the researches (mainly as the COICOP element, e.g. **Dudek and Koszela, 2013** or **James, Lomax and Birkin, 2019**), the connection of the consumption of food and degree of urbanization stays unique.

CONCLUSION

Tendencies towards urbanization are observable all over the world. The increased role of cities is evident also in the EU. This paper examines the consumption of the COICOP class – food and non-alcoholic beverages by the degree of urbanization on the sample of EU countries. As data shows, the share of an examined class of COICOP in total consumption of cities, towns, and suburbs and rural areas presents the second largest item of the total consumption of all structures in question.

This paper examines two scientific hypotheses. First, the consumption of food and non-alcoholic beverages increases with the decrease of urbanization degree. For this purpose, data visualization is employed. Maps are created based on scaled consumption of food and non-alcoholic beverages in cities, towns, and suburbs and rural areas in the three periods – 2005, 2010, and 2015. Nine maps are created. The spatial distribution of data shows that higher shares of consumption of food and non-alcoholic beverages are obtained from CEE and southern countries in all structures and all periods. Considering that consumption of food and non-alcoholic beverages is negatively correlated with GDP per capita or household expenditure per capita, it is possible to conclude that countries with lower levels of GDP per capita spend more on goods of daily use. Finally, in this case the hypothesis has been confirmed. In cities, the share of consumption of food and non-alcoholic beverages in total consumption is lower in comparison with other structures.

Second, the scientific hypothesis stresses the potential change of similarities between EU countries in the consumption of food and non-alcoholic beverages by the degree of urbanization in respectthe of recent financial crisis. To this purpose, the cluster analysis is provided in the research based ons of k-means clustering, similarities between EU countries in the consumption of food and non-alcoholic beverages by the degree of urbanization and with respect to socio-economic conditions are investigated. However, the inter-period comparison of cluster constitution (clusters are made for the all three monitored periods) is made. Obtained results show that similarities between EU countries in consumption of food and non-alcoholic beverages by the degree of urbanization change through the time. Also in this case the scientific hypothesis has been confirmed. The delayed effect of financial crisis may explain observed changes. Obvious relocation of countries is evident when comparing clusters in period 2010 and 2015. Besides it, the most stabile cluster is a cluster, which contains core EU countries (Germany, France, Italy and the United Kingdom).

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SHELF LIFE OF TEMPEH PROCESSED WITH SUB-SUPERCRITICAL CARBON DIOXIDES

Maria Erna Kustyawati, Filli Pratama, Daniel Saputra, Agus Wijaya

ABSTRACT

Tempeh, a fermented soybean-based food originally from Indonesia, is a remarkably nutritious functional food with health benefits. Unfortunately, tempeh is highly perishable, with a shelf life of 24 – 48 hours. The goal of this research was to evaluate the possibility of a sub-supercritical CO_2 technique to increase the shelf life of tempeh by measuring the changes in the L^* (lightness) value and texture of tempeh via application of a kinetic approach and, based on the observations, to estimate its shelf life. Tempeh was processed with sub-supercritical CO_2 at 6.3 MPa for 10 min, then together with unprocessed tempeh (control), stored for 5 days at temperatures of 20, 30 and 40 °C. The Accelerated Self-Life Test (ASLT) with the Arrhenius model was used to measure the shelf life of processed and control tempeh. The calculated shelf life of processed tempeh using the ASLT by the Arrhenius method was 2.43 days at 20 °C, 3.7 days at 30 °C and 1.4 days at 40 °C, and the shelf life of unprocessed tempeh was 3.33 days at 20 °C, 2.90 days at 30 °C and 2.56 days at 40 °C. The conclusion was that the use of sub-supercritical CO_2 at 6.3 MPa for 10 min increased the shelf life of tempeh stored at 30 °C.

Keywords: sub-supercritical CO_2; kinetic change; shelf life; tempeh

INTRODUCTION

At present, consumers demand fresh food that is not only of high quality and safe but also has a long shelf life. High-pressure carbon dioxide (HPCD) technology has been developed as a food processing technology with the advantage of minimizing the loss of heat-sensitive nutrients. Carbon dioxide in the supercritical state has the dual properties of a gas with high diffusivity and a liquid with high solubility **(Ferrentino et al., 2010)**. These properties allow HPCD to diffuse easily through complex matrices, causing modification in either macromolecular or micromolecular substrates **(Garcia-Gonzales et al., 2007; Liao et al., 2010; Ferrentino, Balzan, and Spilimbergo, 2012; Guo et al., 2011)**. Many researchers have shown that HPCD could extend the shelf life of food by killing microbes and enzymes at a relatively low temperature whilst preserving the nutritional and sensory qualities of vegetables and food products. HPCD could inactivate *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* **(Bourdoux et al., 2018; Liao et al., 2010)**, as well as the natural microbial flora **(Li et al., 2012; Cappelletti et al., 2015)**. HPCD has also been proven capable of inactivating the enzymes that cause food spoilage and lowering food quality, such as pectin methyl esterase, poly galacturonase, peroxidase, polyphenol oxidase and lipoxygenase **(Niu et al., 2019; Illera et al., 2018; Briongos et al., 2016; Liu et al., 2010; Hu et al., 2013)**.

Tempeh is an Indonesian fermented food made from soaked, hulled and cooked soybeans inoculated with the fungus *Rhizopus oligosporus*. Tempeh is a remarkably nutritious functional food with health benefits; however, it is highly perishable, with a short shelf life of 36 – 48 hours at room temperature **(Sparringa and Owens, 1999; Nout and Kiers 2005; Djunaidi et al., 2017)**. Several researchers have reported on methods of extending the shelf life of tempeh. Frozen storage (-18 °C) of tempeh resulted in loss of taste and texture which softened after thawing, while refrigerated storage caused discoloration and spoilage after 72 hours **(Witono et al., 2015)**. Meanwhile, tempeh kept under modified atmosphere packaging (15% O_2, 30% CO_2 and 55% N_2) spoiled in 24 hours **(Muslikhah, Anam and Andriani, 2014)**. A previous study performed by the author found that processing of tempeh with high pressure CO_2 at a pressure of 6.3 MPa for 10 min reduced the number of bacteria, yeasts and moulds in tempeh to 4.1, 5.1 and 4.3 log CFU g^{-1}, respectively. This discovery led us to study the effect of HPCD on the shelf life of tempeh. The objective of this research was to study the degradation kinetics of the quality parameters of tempeh processed with sub-supercritical CO_2 and to determine the shelf life of such tempeh.

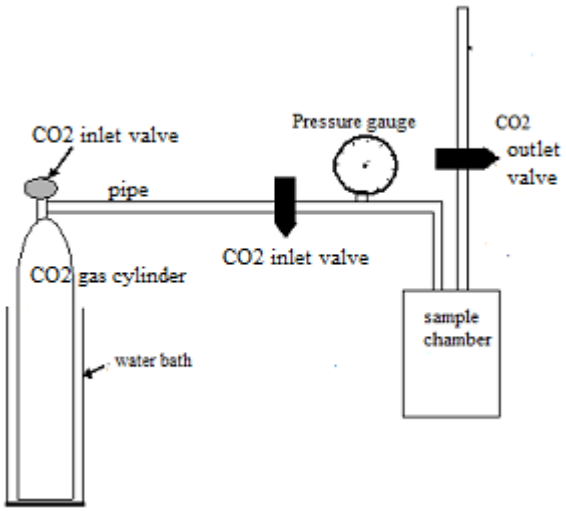

Figure 1 The set-up of the experimental apparatus **(Saputra, 2006)**.

Scientific hypothesis

The shelf life of tempeh stored at 20, 30, and 40 °C can be extended by treatment with CO_2 at sub-supercritical pressure.

MATERIAL AND METHODOLOGY

Treatment of tempeh with supercritical CO_2

Tempeh, in the form of a cylinder with a diameter of 35 mm and a length of 100 mm, fermented for 36 hours at 30 °C, was obtained from the Center of Home Industry Tempeh Making Palembang, Indonesia, placed in a cooler box and carried to the laboratory for direct processing **(Kustyawati et al., 2018)**.

The high-pressure CO_2 installation used for experimental treatments, consisting of a CO_2 gas cylinder, a cylindrical pressure chamber, pressure gauges, and a water bath at a constant temperature, is shown in Figure 1 **(Saputra, 2006).** Fresh tempeh was placed in a pressure chamber and then closed tightly. When the designated temperature in the water bath reached a constant 25 °C, and all pipe connections were secured, commercially available CO_2 was injected through the gas inlet valve from the gas cylinder into the pressure chamber, within 1 minute, until it reached the desired pressure of 6.3 MPa (sub supercritical CO_2 condition), which was indicated by the pressure gauge. After 10 min of treatment with sub-supercritical CO_2, the pressure was lowered to atmospheric pressure within 2 minutes by slowly opening the gas outlet valve. The tempeh was then collected aseptically from the pressure chamber using sterilized tongs, placed in a sterilized container, and stored in a refrigerator for further study. The processed and unprocessed tempeh (control) were analysed further for colour and texture, before and during the storage period.

Storage study

Tempeh processed with sub-supercritical CO_2 (6.3 MPa, 25 °C for 10 minutes) and unprocessed tempeh were employed as the treatment and control, respectively, in this experiment. All of the tempeh was stored for 5 days. Tempeh was stored as follows: tempeh samples were placed on a Styrofoam plate and covered with plastic film then stored at 20, 30 and 40 °C with the same relative humidity. Observations on quality parameter changes (*C*) were carried out by measuring the quality attributes represented by *L** and texture. Observations were made daily. A storage time of 5 days was chosen considering that the shelf life of fresh tempeh is normally around 24 – 48 hours at room temperature (28 – 30 °C).

The Accelerated Self Life Test (ASLT) with the Arrhenius model was used to determine the shelf life of tempeh, in which, if the food product deteriorates faster, then the shelf life is determined based on extrapolation to storage temperature. Changes in the quality factor were used to determine the degree of decrease in quality. Data were transformed into a kinetic plot, and an appropriate kinetic parameter model was obtained. The quality decrease in food is given by equation (1).

$$\frac{dQ}{dt} = k.Q^n \qquad (1)$$

Where: Q is the quality factor, t is time, k is a rate constant that depends on temperature, n is a degree factor or reaction order and $dQ\ dt^{-1}$ is the change in the Q factor per unit of time.

Most of all, a decrease in food quality includes zero-order (order 0) and first-order (order 1) reactions. The Arrhenius correlation chart was generated by evaluating the rate constant (k) at three different temperatures. The rate constant (k) was predicted by extrapolating the correlation between $ln\ k$ and 1/T at three temperatures. Shelf life is determined on the basis of the most influential factors on the product. One of the factors that can affect a product's shelf life is temperature. The Arrhenius kinetic approach was used to determine the shelf life and temperature limit factor. The equation for the Arrhenius model is shown in equation (2).

$$k_T = k_o e^{\frac{-E_a}{RT}} \qquad (2)$$

Where: k_T was the reaction rate constant of quality degradation, k_o was a constant (frequency factor, not dependent on temperature), E_a was activation energy, T was absolute temperature (K) and R was the gas constant (8.341 $J.mol^{-1}.K^{-1}$).

The zero-order and first-order quality reaction was measured by using equation (3) and equation (4), respectively **(Labuza and Szybist, 2001)**.

Zero order: $$t = \frac{(C_1 - C_o}{k} \quad (3)$$

First order : $$t = \frac{ln\frac{C_1}{C_o}}{k} \quad (4)$$

Where: C_o was the initial quality value of storage, C_t was the quality value at the storage time t, k was the reaction rate constant and t was the storage time (days). The determination of the order of the most suitable reaction was performed by selecting the equation with the highest R^2.

Colour and texture measurement

The surface colour analysis of tempeh was evaluated as the *CIE L*a*b** value and *LCH* colour scale using a colour difference meter (TC-1500, Tokyo, Japan). Results were expressed as L^* (Lightness), a^* (redness) and b^* (yellowness).

The total colour difference (ΔE^*) between the control and the processed tempeh was obtained using the following equation (5):

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (5)$$

Where: ΔL^*, Δa^* and Δb^* were the differences between L^*, a^* and b^* after treatment and the L^*, a^* and b^* values of the standard colour. The standard colour used in this experiment was the L^*, a^* and b^* values of the unprocessed tempeh (control), which was L^*=76.6, a^*=3.1 and b^*=7.5.

Texture analysis

The texture analysed was tempeh hardness. The greater the value, the harder the sample being analysed. The LFRA Texture Analyzer (Brookfield AMETEK CT3-100-115), type A 7.1 was used to measure tempeh texture. The texture of the tempeh in this study was the quality of compactness of tempeh when sliced, because compact and dense soybean strands produce tempeh that is easily sliced. Tempeh has a non-homogeneous texture because it consists of woven soybean seeds arranged mycelia. This arrangement gives rise to varying angles/areas of penetration of the probe, for example, the possibility of probes piercing right into the soybean seeds or the soft areas between soybeans strands. Therefore, a Brooke-type probe blade was used in this study. The Brooke-type blade presses right in the centre of the sample. The peak load and final load numbers in units of gram force (gf) listed on the display were recorded. Measurements were performed in three replicates.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel 2003 and Statistica 8.0 StatSoft software. Analysis of variance (ANOVA) was used to study differences between samples. A software program using Duncan's multiple range test was used to compare treatment means. A value of p <0.05 was considered statistically significant. All experiments were performed with at least three replicates.

RESULTS AND DISCUSSION

Kinetics of quality degradation

Processed and unprocessed (control) tempeh were used as the models in this experiment. Colour is very important to the sensory nature of tempeh because it is the first characteristic observed by the consumer. The colour of tempeh produced by the growth of mould was influenced by changes in the chemical composition of the tempeh and storage temperature. The lightness (L^*) value of the control was high, approximately 75.7 on the initial day, and then the lightness darkened to 57 at the third day of storage. The initial L^* value of the tempeh processed with sub-supercritical CO_2 glimmered to 74.4 and decreased to dark (69.3) by the fifth day (Table 1 and Table 2).

The data obtained from the experiments were plotted on a graph of the relationship between the degradation in the quality of L^* and texture, and the storage time at various temperatures. Based on the correlation coefficient (R^2) of texture and L^* (Table 3), the rate of change in the quality of tempeh followed the first-order reaction model. A higher correlation value indicates a faster decline in reaction product quality. This was in agreement with the findings of **Ahmed, Shivhare and Raghavan (2001)** that the degradation of betanin, a natural colour compound in beets induced by heat, followed a first-order reaction.

Figure 2 shows that the lightness value (L^*) weakened during storage at various temperatures, where it developed from light to dark. The colour of fresh tempeh is brownish yellow, due to the compounds furosine, hydroxymethylfurfural (HMF) and acrylamide, which are the products of the Maillard reaction in beans **(Zilic et al., 2014)**. Tempeh is made from cooked soybeans which have been heated to boiling temperature. In addition, during the depressurization process of the high-pressure CO_2 treatment, the mycelia of the mould are wiped from the surface of the tempeh, resulting in a brownish-yellow colour appearing on the beans. Moreover, a study performed by **Handoyo and Morita (2006)** found that over-fermentation of tempeh that occurred during storage could bring about protein depletion and produced a blackish-brown colour.

The hard texture of tempeh increased significantly during storage (p <0.05) (Table 1 and Table 2). The texture of tempeh is dense, compact and sliceable. It is formed by soybean cotyledons intertwined with the mycelia of moulds. As mould grows, it produces fluffy white mycelia which bind the beans, squeezing and penetrating the cell walls to create a cake texture and simultaneously producing enzymes that cause softening of the beans due to hydrolysis of various compounds during fermentation **(Duniaji et al., 2019; Jones et al., 2017; Wati et al., 2020)**.

Table 1 The lightness (L^*) and texture of processed tempeh during storage at 20, 30 and 40 °C.

20 °C			30 °C			40 °C		
Day	***L****	**Texture**	**Day**	***L****	**Texture**	**Day**	***L****	**Texture**
0	74.6 ±0.06 ^{a}	577 ±0.1^{a}	0	74.4 ±0.05^{a}	577 ±0.3^{a}	0	74.6 ±0.05^{a}	577 ±0.2^{a}
1	74.1 ±0.07^{a}	580 ±0.2^{ab}	1	73 ±0.050^{ab}	579 ±0.1^{ab}	1	69.3 ±0.06^{b}	701 ±0.2^{b}
2	73 ±0.02^{ab}	585 ±0.1^{b}	2	71.5 ±0.03^{b}	610 ±0.1^{b}	2	62.3 ±0.06^{bc}	789 ±0.3^{c}
3	71.4 ±0.03^{b}	589 ±0.1^{bc}	3	69.3 ±0.06^{bc}	625 ±0.3^{bc}	3	58.2 ±0.06^{bc}	860 ±0.23^{cd}
4	70 ±0.03^{bc}	592 ±0.3^{c}	4	68.4 ±0.06^{bc}	660 ±0.3^{c}	4	56 ±0.05^{c}	870 ±0.4^{cd}
5	69 ±0.10^{c}	593 ±0.3^{c}	5	66 ±0.07^{c}	690 ±0.2^{d}	5	55.5 ±0.07^{c}	885 ±0.1^{d}

Note: All values are the mean and standard deviation of three replicates. $^{a-d}$ Means within a column with different letters were significantly different (p <0.05).

Table 2 The lightness (L^*) and texture of control (unprocessed tempeh) during storage at 20, 30 and 40 °C.

20 °C			30 °C			40 °C		
Day	***L****	**Texture**	**Day**	***L****	**Texture**	**Day**	***L****	**Texture**
0	75.3 ±0.11^{a}	505.7 ±1.0^{a}	0	75.3 ±0.09^{a}	506 ±0.09^{a}	0	75.3 ±0.9^{a}	505.5 ±0.1^{a}
1	70.8 ±0.58ab	346.5 ±1.0^{b}	1	74.2 ±0.08^{a}	396 ±0.05^{b}	1	69.5 ±1.0^{ab}	1233 ±0.09^{b}
2	66.7 ±0.11b	332 ±0.9c	2	69.5 ±0.1^{b}	286 ±0.1^{cd}	2	65.2 ±1.1^{b}	1321 ±0.04^{c}
3	63.2 ±0.12bc	338 ±0.9cd	3	65.7 ±0.12^{c}	305 ±0.1^{c}	3	60.7 ±1.2^{c}	1442 ±0.04^{cd}
4	59.5 ±0.09^{c}	340 ±0.7cd	4	60.4 ±0.11^{cd}	290 ±0.09^{cd}	4	55.3 ±1.1^{d}	1467 ±0.02^{cd}
5	58 ±0.09^{c}	300 ±1.0^{d}	5	57 ±0.8^{d}	277 ±0.1^{d}	5	54 ±0.9^{d}	1511 ±0.02^{d}

Note: All values are the mean and standard deviation of three replicates. $^{a-d}$ Means within a column with different letters were significantly different (p <0.05).

Table 3 Evaluation of the linear regression equation for the estimated shelf life of tempeh.

Quality parameters		**T, °C**	**Zero order**	***R²***	**First order**	***R²***
			Regression equation		*Regression equation*	
PT (Processed Tempeh)	*L**	20	y= -1.197x+75.01	0.982	y=0.0167x+4.318	0.981
		30	y= -1.657x+74.57	0.990	y=0.0236x+4.312	0.988
		40	y= -3.985x+72.61	0.919	y=0.0625x+4.287	0.933
	Texture	20	y=3.428x+577.4	0.970	y=0.0059x+6.359	0.969
		30	y=23.51x+564.7	0.959	y=0.0375x+6.339	0.965
		40	y=60.51x+629.0	0.877	y=0.0821x=6.443	0.847
CT (Control Tempeh)	*L**	20	y=-3.54x+74.43	0.983	y= -0.0537x+4.313	0.989
		30	y=-3.906x+76.78	0.980	y= -0.059x+4.347	0.975
		40	y=-4.388x+74.31	0.981	y= -0-069x+4.314	0.986
	Texture	20	y=-29.76x+434.7	0.581	y=0.0757x+6.061	0.615
		30	y=-41.25x+446	0.722	y= -0.111x+6.090	0.745
		40	y=167.1x+828.6	0.687	y= -0.174x+6.634	0.599

Shelf-life prediction

The influence of temperature on the reaction rate was described using the Arrhenius equation. The regression equation and value of the R^2 data for tempeh at 20, 30 and 40 °C are shown in Table 3. The lightness (L^*) decreased faster than the texture, which was indicated by the slope values. Plotting *ln k* against *1/T* produced a linear regression of the Arrhenius model in which the slope represents the E_a value (Table 4). The E_a values of L^* and texture were 12.27 and 25.59 kcal.mol^{-1}, respectively, indicating that lightness was more sensitive to temperature. The sensitivity of quality parameters to changes in temperature can also be evaluated based on the value of the correlation coefficient R^2, where the greater the value of R^2, the greater the relationship between changes in the rate constant (*k)* and temperature. The dates on which characteristic limits for processed tempeh were attained with respect to lightness characteristic criteria were 2.43, 4.88 and 9.36 days at storage temperatures of 20, 30 and 40 °C, respectively, while those with respect to texture characteristic criteria were 8.6, 3.7 and 1.4 days at storage temperatures of 20, 30 and 40 °C, respectively. The shelf life was defined as the earliest date of all the dates on which characteristic limits were attained when each characteristic criterion reached its limit. Therefore, the shelf life of processed tempeh was estimated to be 2.43 days at 20 °C, 3.7 days at 30 °C and 1.4 days at 40 °C. The dates on which characteristic limits were attained for the control (unprocessed tempeh), with respect to lightness characteristic criteria, were 3.33 days, 2.90 days, and 2.56 days at 20, 30 and 40 °C, respectively, while those with respect to texture characteristic criteria, were 6.89 days, 4.47 days and 2.99 days at 20, 30 and 40 °C, respectively. Therefore, the shelf life of unprocessed tempeh was estimated to be 3.33 days at 20°C, 2.90 days at 30 °C and 2.56 days at 40 °C. Summarizing the results, the shelf life processed tempeh was longer than that of unprocessed tempeh at 30 °C. However, the shelf life estimated in this study cannot be applied to all tempeh, because many factors including consumer palatability and consumer perspective, also play vital roles.

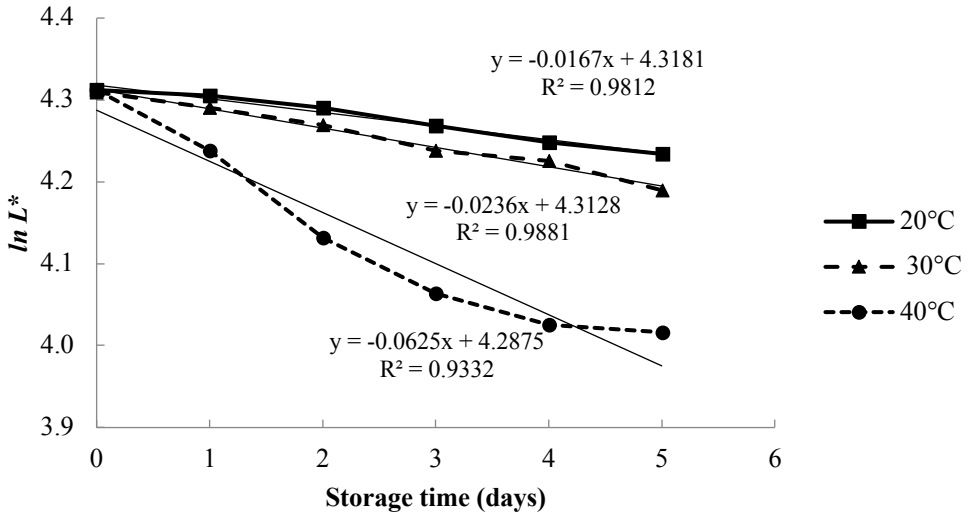

Figure 2 The correlation between lightness (*L**) of processed tempeh and time according to a first-order reaction.

Table 4 Regression equation of tempeh stored at 20, 30 and 40 °C.

	Quality parameter	T, °C	Regression equation	Reaction order	E_a, [1] kcal.mol-1	K,[2] day-1	R^2	C_o[3] - C_t[4]	Shelf - life (days)
PT[5]	Lightness (*L**)	20	LnK= -6177.2x+16.839	First	12.27	0.292	0.923	5.1	2.43
		30				0.145			4.88
		40				0.758			9.36
	Texture	20	LnK= -12883x+38.85	First	25.59	0.938	0.950	48	8.60
		30				0.218			3.67
		40				0.559			1.42
CT[6]	Lightness (*L**)	20	LnK= -1206.8x+1.1719	First	2.40	0.053	0.984	9.6	3.33
		30				0.060			2.90
		40				0.068			2.56
	Texture	20	LnK= -3827.6x+10.458	First	7.60	0.073	0.995	201	6.89
		30				0.113			4.47
		40				0.169			2.99

Note: [1] Activation energy in kcal.mol^{-1}; [2] Rate constant; [3] Initial value of quality parameter; [4] Data of quality parameter as t time passes; [5] Processed tempeh; [6] Control (unprocessed tempeh).

All food expiration dates could be established as self-applied safety factors by each producer. For tempeh, expiration dates may not be mandatory because tempeh categorized as a fresh food product has a very short shelf life, and the spoilage of tempeh is easily detectable by looking at the colour, texture and aroma. Therefore, an expiration date is not necessary. From the point of view of microbial safety, tempeh, fermented soybean, is a reliably safe food because bacteria, yeasts and moulds that grow in tempeh have their own specific role. *R. oligosporus*, an important fungus in tempeh, is known to produce antibiotics against bacteria **(Kobayasi, Okazaki and Koseki, 1992; Wang et al., 1969)**. *Bacillus subtilis*, the most common bacteria in tempeh, contribute to the production of fatty acids and isoflavones **(Barus et al., 2017; Kanghae, Eungwanichayapant and Chukeatirote, 2017)**. The role of yeast in tempeh is not clear **(Nout and Kiers, 2005; Pleva et al., 2018)**; however, the authors' previous results show that co-culturing *Saccharomyces cerevisiae* with *R. oligosporus* in soybean fermentation produced tempeh with a pleasant yeast/tapai aroma that was liked by panellists **(Kustyawati, Nawansih and Nurdjanah, 2017)**. Further studies on the role of yeast in tempeh production are needed.

CONCLUSION

Sub-supercritical CO_2 processing at 6.3 MPa for 10 min increased the shelf life of tempeh at a storage temperature of 30 °C. The shelf life of processed tempeh was 2.43 days at 20 °C, 3.7 days at 30 °C and 1.4 days at 40 °C, and the shelf life of unprocessed tempeh was 3.33 days at 20 °C, 2.90 days at 30 °C and 2.56 days at 40 °C.

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IMPACT OF HUMIC ACID AS AN ORGANIC ADDITIVE ON THE MILK PARAMETERS AND OCCURRENCE OF MASTITIS IN DAIRY COWS

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ABSTRACT
Given growing concerns about the use of antibiotics in the treatment of animals, identifying organic alternatives as feed additives to improve animal health and the development of immune responses has become of interest in dairy farming. Humic acids (HA) seem to be a suitable alternative with a favorable impact on the health and production parameters of animals. This study aimed to determine the effects of an HA supplemented diet on milk parameters as well as the effects on somatic cell count (SCC) and the occurrence of mastitis in dairy cows during the peripartum period. Twenty dairy cows in the last stage of pregnancy were selected from a herd of 140 cows. The selected cows were randomly divided into two groups: control (C) and experimental (E). The two groups were fed the same feed mixture and group E was additionally supplemented with HA at a total dose of 100 g per cow per day during the last 50 days of pregnancy. The milk parameters (dry matter, lactose, fat, crude protein, casein and milk urea) and SCC of every cow, and the presence of mastitis, were checked on days 10 and 30 during the first month of lactation. The results of the study show that dietary supplementation with HA significantly reduced the milk urea (MU) content and SCC on the 10th day after calving but did not affect the other milk compositions. In addition to the decreased MU and SCC, the number of positive quarters detected by the California Mastitis Test was reduced by 20.0% and the occurrence of mastitis caused by coagulase-negative staphylococci (CNS). Based on the obtained results we can conclude that the addition of HA stabilizes the nutrient digestion, as was confirmed by a reduced MU content in the supplemented group. Their indirect beneficial effects improved the development of immune responses, resulting in decreased SCC and the occurrence of mastitis caused by CNS.

Keywords: dairy cows; supplementation; humid acid; mastitis; milk urea

INTRODUCTION
The health-safety and nutritional quality of raw milk are influenced by many factors. One of the main factors that affect the health of and milk production by dairy cows is mastitis. Mastitis is an inflammation of the mammary gland characterized by physical, chemical, bacteriological, and cytological changes in the milk. Changes in the quality and quantity of milk, as well as pathological changes in the glandular tissues of the udder have been observed **(Pyörälä and Taponen, 2009)**.

Mastitis is mainly caused by microorganisms. These are usually bacteria, including gram-negative and gram-positive bacteria, mycoplasmas, yeasts, and algae **(Zadoks et al., 2011)**.

The majority of mastitis cases are caused by a few common bacterial pathogens involved: *Staphylococcus* spp. (*S. aureus, S. warneri* and *S. chromogenes*), *Streptococcus* spp. (*Str. agalactiae, Str. dysgalactiae, Str. uberis* and *Str. bovis*), coliforms (mainly *E. coli* and *Klebsiella pneumoniae*) and *Actinomyces pyogenes* **(Idriss et al., 2013)**. Although some pathogens from the group of coagulase negative staphylococci (CNS) and *Corynebacterium bovis*, are historically considered to be of limited importance and are therefore often described as minor pathogens. In the last decade the impact of CNS has increased probably because the prevalence of major pathogens has decreased **(Pyörälä and Taponen, 2009)**.

Mastitis and other diseases are common problems in dairy herds, resulting in increased costs and decreased production. Most diseases in dairy cows occur at or just after calving, which is a period associated with immune suppression, resulting in increased susceptibility to infections. Prepartum immune suppression is multifactorial but is associated with endocrine changes and destabilization of intestinal flora, leading to impaired digestion and utilization of nutrients from animal feed **(Xiaowang, Shaohua and Lixia, 2010; Zigo et al., 2014)**.

During the past few decades the use of organic feed additives to improve health, wellbeing, and production has been investigated in some areas of animal husbandry. Humic substances (HS) are one such additive **(Marcinčáková et al., 2015; Semjon et al., 2020)**.

Humic substances (HS) are geological deposits made of a complex mixture of acids that arise from the natural decomposition of plant and animal material by soil microorganisms occurring in water, soil, carbon and other sources. They are heterogeneous high molecular weight organic substances and their composition differs according to the geographic region **(Jaďuttová et al., 2019; Mudroňová et al., 2020)**.

A yellow to brown-coloured seam (brown seam) may contain high concentrations of fulvic acid, whereas a dark brown to black-coloured seam (black seam) may contain high amounts of humic acid and humin. Humic acids (HA) are considered to be adsorbent, because of various binding sites present in their structure. It has been assumed that humic acids could reduce the absorption and systemic availability of bacterial endotoxins, which could be of great importance in the protection of animal and human health **(Trckova et al., 2005; Galip, Polat and Biricik, 2010)**.

Moreover, many positive effects on the performance and health of animals have been attributed to humic acids. They inhibit the growth of pathogenic bacteria and moulds and decrease the level of mycotoxins and thus may lead to improved gut health **(Marcinčáková et al., 2015)**.

Humic acids stabilize the intestinal flora, and in this way, improve the utilization of nutrients from animal feed, which affects the composition of dairy cows' and goats' raw milk **(Potůčková and Kouřimská, 2017)**.

Scientific hypothesis

Previous studies reported that the addition of HA to the diet of cows stimulated the fermentation products with improved nutrients digestion, growth and development of immune responses, but there is no data on the effect of its use on cow milk parameters and mammary health. Therefore, the aim of this work was to evaluate the effects of a humic acid supplemented diet on the main milk parameters and composition as well as the occurrence of mastitis in dairy cows during the peripartum period.

MATERIAL AND METHODOLOGY

Animal care

The practical part of the study was carried out in a dairy herd of 140 crossbred Slovak Pied cattle x Red Holstein. Dairy cows from the monitored herd were kept in a free housing system with a separate calving barn, equipped with individual boxes with bedding and were allowed ad libitum access to water. Cows were cow according to the Nutrient Requirements of Dairy Cattle **(NRC, 2001)**.

During the lactation, period cows were milked twice a day at 4:30 a.m. and 4:30 p.m. in the fishing-milking fed twice a day with feed mixture formulated for a 650 kg parlour (FarmTec) 2x10 pcs (Figure 1). First, water was used to remove impurities from the udder and teats. Subsequently, the udder was thoroughly wiped with disposable paper wipes. The first milk from each quarter was hand-drawn into a dark-bottomed pot, and the milk was subjected to sensory analysis. During the milking process, the pulsation ratio was 60:40 at a rate of 52 $c.min^{-1}$ and milking was automatically terminated when the milk flow dropped to 0.2 $L.min^{-1}$. After milking, the teats were disinfected by teat-dipping. Before drying an intramammary antibiotic preparation Orbenin Dry Cow a.u.v. (Pfizer, IT) was applied to every quarter of the udder in pregnant cows.

Experimental design, animals and diets

The experimental conditions were designed in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching **(FASS, 2010)**. Twenty gravid cows in the last stage of pregnancy were selected from the herd. Fifty days before the expected calving date the cows were randomly divided into two groups, control (C) and experimental (E). The 10 animals per group were housed on a deep litter divided into two separate stables with *ad libitum* access to water and feed.

Selected cows from each group were fed twice a day with a total mixed ration (TMR) containing corn silage (65%), grass hay (12%), barley straw (10%), bean (11.7%) and concentrate (1.3%) with the content of extracted rape and soy meal according to the current **NRC (2001)** during the dry period (Table 1). The mean daily intake for the dry period under study was 9.6 kg of DM per cow per day.

The experimental group (E) was supplemented into the diet with humid acids at a dose of 100 g per cow per day. The humic acids (product Humac Nature AFM) used in the experiment were obtained from Humac ltd. the company, SR. According to the producer, Humic Nature as an organic additive in the diet of animals contains: total humic acids 65% and minerals 15%, of which accounted for free humic acids 60%.

The experimental period lasted 50 days before the expected parturition and ended immediately after calving. Subsequently, the calves were separated from cows that were then milked into individual containers. Five days after calving the cows from the control and experimental group were milked twice a day together with all lactating cows.

Udder health examination and milk sampling

Udder health was evaluated and milk samples were taken from each selected cow on days 10 and 30 of the first month of lactation. A thorough evaluation of udder health included clinical examination, sensory analysis of milk from forestripping of each udder quarter followed by the assessment of CMT (Indirect Diagnostic Test, Krause, Denmark). Milk from every quarter was mixed with the reagent and the result was scored as negative, trace, or positive (score 1 – 3) depending on the formation of gel in the milk sample according to **Jackson and Cockroft (2002)**.

Next, we collected a milk sample from one quarter for bacteriological cultivation and two mixed milk samples for measurement of the milk components and SCC from each cow aseptically in accordance with the guidelines of the **National Mastitis Council (2001)**. The samples were cooled to 4 °C and immediately transported to the laboratory and analysed on the following day.

Analytical methods

TMR chemical composition

A 1 kg sample of TMR was analysed for dry matter (DM), crude protein, crude fat, ash neutral detergent fibre

(NDF) and acid detergent fibre (ADF) according to **AOAC** methods **(2012)**. The net energy (NE) contents were obtained by calculation **(NRC, 2001)**.

Determination of milk parameters

The raw milk samples were analysed for dry matter (total solids), non-fat dry matter (SNF; solids non-fat), lactose, fat, proteins content and pH using the Milk analyzer Lactoscan MCCW (Milkotronic, Bulgaria) according to **Potůčková and Kouřimská (2017)**. Milk urea (MU) content was measured on a CHEMSPEC apparatus (Bentley Instruments Inc.) according to **Pecka et al. (2012)**. All measurements were performed twice for each sample.

Determination of SCC

The somatic cell count (SCC) is one of the internationally recognized standards for milk quality control and is also a useful indicator of mastitis presence.

The Somatic Cell Counter Lactoscan SCC (Milkotronic, Bulgaria) is based on direct fluorescent, low magnification microscopic somatic cell counting. Lactoscan SCC uses a very sensitive fluorescent dye (Sofia Green) and LED optics (CCD technologies) in order to make the cell analysis more accurate, reliable and fast.

Laboratory analyses

Bacteriological examinations were performed according to commonly accepted rules **(Malinowski et al., 2006)**. Milk samples (10 μL) were cultured at the respective veterinary practice according to routine procedures, usually employing Columbia Blood Agar Base with 5% of defibrinated blood, Staphylococcal medium N° 110, Baird-Parker agar, Edwards Medium, Mac Conkey Agar (Oxoid, OXOID Ltd., Basingstoke, Hants, UK), and incubation at 37 °C for 24 h.

As well as evaluating bacterial growth characteristics other assays were used to identify bacterial species: pigment and coagulase production, catalase activity, haemolysis, Gram staining and other virulence factors. *Staphylococcus* spp. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspected colonies of *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriacae* spp. were isolated on blood agar and cultivated at 37 °C for 24 h and identified biochemically using the Staphy test, Strepto test and resp. Entero test using the software TNW Pro 7.0 (Erba-Lachema, CZ) according to the manufacturer's instructions.

Statistical analysis

A one-way ANOVA with an F-test on the arithmetic means (M) from 10 parallel measurements with standard deviation (SD) of dry matter (total solids), SNF (solids non-fat), lactose, fat, protein content, pH, MU and SCC was performed by Microsoft Excel 2003. Statistical significance was set at $p < 0.05$. The differences in the prevalence of mastitis and distribution of bacterial pathogens among monitored groups of cows were statistically analysed using the Chi-square test. The dependence of the individual signs was tested at a significance level $\alpha = 0.05$, with critical value = 5.991.

RESULTS AND DISCUSSION

Table 2 illustrates the effect of the supplementation of HA on the milk components and SCC in dairy cows. The two groups of animals, control and experimental, were fed with TMR (Table 1) during the 50 days prior to parturition. In addition to this feed, the experimental group was supplemented with HA at a dose of 100 g per cow per day. Changes of MU content (Figure 2) and SCC on the 10th day after calving in HA supplemented group was reported. No changes in the composition of dry matter, SNF, lactose, fat, protein content, or pH in raw milk were noted during the monitoring period (Table 2).

The observed decreased level of MU in cows fed diet with HA can be explained by lower blood urea nitrogen (BUN) level which resulted from lower ruminal ammonia nitrogen (NH_3-N) concentration indicated by more efficient utilization of dietary crude protein (CP).

The same trend was observed in the study by **Van Soest (1994)** who supplemented dairy cows with HA during lactation. In HA treated cows, lower blood urea nitrogen (BUN) value was indicated by more efficient utilization of dietary CP for microbial protein synthesis associated with nitrogen-binding capabilities of HA. This low value of BUN is usually associated with a lower ruminal NH_3-N flux to the bloodstream. Thus, a reduction in MU result can be explained with the reduction of BUN supported by lower ruminal NH_3-N concentration.

Similar results were observed by **Degirmencioglu (2014)** after supplementation of HA to ruminants for 90 days of lactation, with no improvements in milk composition (non-fat dry matter, lactose, fat and protein content). The effect on milk yield was inconsistent.

Other studies have reported positive effects of HA on milk production, milk fat **(Thomassen and Faust, 2000)** and milk protein **(Potůčková and Kouřimská, 2017)** in dairy cows. Another study showed that the use of HA as an animal feed supplement leads to increased milk production and increased butterfat percentage in dairy cows **(Islam, Schumacher and Gropp, 2005)**.

However, it is difficult to compare the effects of HS across studies due to the different sources and preparations of HA used, as well as because animals reared in various regions of the world are exposed to different climates and environmental conditions.

Milk SCC is a useful tool for measuring milk quality, the health status of the mammary gland and changes in milk composition. In the European Union the legal limit for cows is 400 000 cells.mL^{-1} **(Zajác et al., 2012)** and in the USA the legal limit established by the Food and Drug Administration for cows is 750 000 cells.mL^{-1} **(Paape et al., 2007)**.

In our study, the control group shoved increased SCC value above the legal limit on the 10th day after calving (Figure 2). However, the HA supplemented group had lower SCC values on the 10th day after calving (Table 2) and positive quarters according to the CMT (Table 3).

A score of 1 to 3 in the CMT indicates an increased SCC over the legal limit, most commonly caused by the presence of pathogenic bacteria. The penetration of pathogenic bacteria into the teat canal irritates the delicate mammary tissue causing an inflammatory response and changes in milk quality and composition **(Sharma, Singh and Bhadwal, 2011)**.

In particular, differences were found by comparing the positive quarters in both groups with CMT score 1 and 3. According to our results the number of positive quarters detected by the CMT on the 10th day after calving was reduced by 20.0% in the group of cows supplemented with HA (Table 3).

Similar to our study, **Thomassen and Faust (2000)** reported that HA supplementation significantly decreased the SCC in milk. They reported that a diet containing 3 g.HA.kg^{-1} decreased the SCC level in milk dairy by about 50%.

Similarly, **Xiaowang, Shaohua and Lixia (2010)** reported that a lower (by 40.1%) SCC was observed in their humate-supplemented group compared to the control.

Intramammary bacterial invasion occurs immediately after calving and leads to glandular damage in parenchymatous tissue. The glandular tissue damage leads to increased SCC and reduced milk production. The cellular presence in milk is one of the important protective mechanisms of the mammary gland **(Sharma, Singh and Bhadwal, 2011)**.

The addition of HA at a dose 100 g per cow per day in our study had a positive nutraceutical effect that stimulated neutrophil activity, which may protect against bacterial pathogens and reduce mortality during acute bacterial infection. The results show that in addition to reducing SCC the number of positive quarters infected with CNS was reduced by 12.5% during the first 30 days of lactation (Figure 3). On the 10th day of lactation, 15 and 9 quarters were infected in the control and experimental group, respectively. The same trend was observed on the 30th day of lactation. A large proportion of CNS (40%) was noted in the control group from infected quarters.

In recent years CNS have become increasingly important in udder infections. They are normal inhabitants of the skin and teat canal and are frequently isolated from milk samples **(Taponen et al., 2006)**. *S. chromogenes, S. haemoyticus* and *S. warneri* were pathologically important in intaramammary infection with increased SCC. The addition of humates to the feed reduced the incidence of staphylococcal infections by 5%.

According to **Dabovich et al. (2003)** the use of HA increases the body's defenses by stimulating neutrophil activity in response to the onset of inflammation. Testing of milk during field trials often indicates an increase in the number of microbes in the milk, an indication to the dairyman of impending mastitis.

As a result of feeding humates, mastitis cases within the milking herd dropped from an average of 3 to 4 cases daily to 4 cases in a month **(Islam, Schumacher and Gropp, 2005)**.

Table 1 Chemical composition of feed mixture.

Component	Content
DM (g.kg^{-1})	408.8
CP (g.kg^{-1}DM)	53.3
Fat (g.kg^{-1} DM)	14.5
NDF (g.kg^{-1} DM)	182.4
ADF (g.kg^{-1} DM)	129.4
Ash (g.kg^{-1} DM)	38.9
Starch (g.kg^{-1}DM)	40.8
NE^{1}, MJ.kg^{-1}	5.65

Note: DM – dry matter, CP – crude protein, NDF – neutral detergent fibre, ADF – acid detergent fibre, NE^{1} - net energy, obtained by calculation.

Figure 1 Dairy cows fed with TMR, assessment of CMT and milking process.

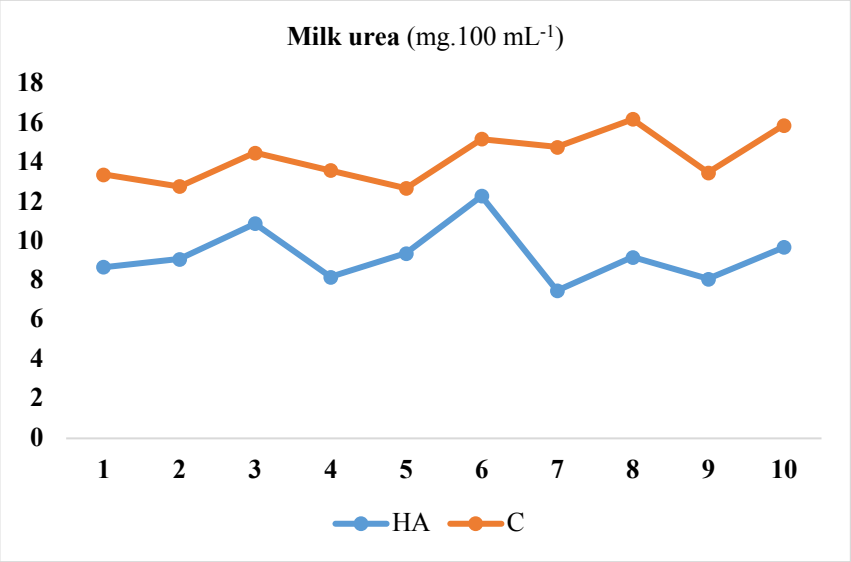

Figure 2 Comparison of milk urea content in supplemented by humic acid (HA) and control (C) group on the 10th day of lactation.

Table 2 Effect of supplemental humic acid on SCC and milk parameters.

Parameters	Groups comparison after 10th day of lactation			Groups comparison after 30th day of lactation		
	Control M ±SD	Experimental M ±SD	*p**	Control M ±SD	Experimental M ±SD	*p**
SCC x 10^3	425.30 ±53.8[a]	358.14 ±41.5[b]	p <0.05	384.42 ±40.02	331.60 ±36.3	p >0.05
MU (mg.100 mL^{-1})	14.26 ±1.21[a]	9.31 ±1.35[b]	p <0.05	12.3 ±1.56	13.61 ±1.42	p >0.05
DM (g.100 g^{-1})	12.68 ±1.23	12.56 ±0.74	p >0.05	12.44 ±0.86	12.68 ±1.13	p >0.05
SNF (g.100 g^{-1})	8.63 ±0.64	8.41 ±0.74	p >0.05	8.32 ±0.55	8.60 ±0.51	p >0.05
Fat (g.100 g^{-1})	4.05 ±0.35	4.15 ±0.56	p >0.05	4.12 ±0.28	4.21 ±0.60	p >0.05
Protein (g.100 g^{-1})	3.47 ±0.36	3.51 ±0.42	p >0.05	3.38 ±0.31	3.55 ±0.38	p >0.05
Lactose (g.100 g^{-1})	4.71 ±0.23	4.90 ±0.18	p >0.05	4.84 ±0.37	4.76 ±0.30	p >0.05
pH	6.61 ±0.09	6.64 ±0.12	p >0.05	6.58 ±0.18	6.65 ±0.16	p >0.05

Note: SCC – somatic cell count; MU – milk urea; DM – dry matter (total solids); SNF – non-fat dry matter (solids non-fat); M – mean; SD – sandard deviation; * p <0.05 – significant difference; * p >0.05 – no significant difference.

Table 3 Milk evaluation per quarter and interpretation of California Mastitis Test (CMT) score.

CMT score	SCC* x 10^3	Interpretation	Evaluated quarters in monitored groups 10th day of lactation		30th day of lactation	
			Control	Exper.	Control	Exper.
N (negat.)	0 – 200	Healthy quarter	37.5	35.0	42.5	47.5
T (trace)	200 – 400 (±50)	Healthy/latent mastitis[1]	25.0	22.5	20.0	22.5
1	400 – 650 (±150)	Subclinical mastitis[2]	20.0[a]	32.5[b]	17.5	12.5
2	850 – 1.200 (±200)	Subclinical/clinical mastitis[3]	10.0	10.0	12.5	15.0
3	1.500 – 5.000 (±300)	Clinical mastitis	7.5[a]	0.0[b]	7.5	2.5

Note: N (neg.) – negative CMT score (healthy quarters); Exper. – experimental group supplemented with HA; [ab]Significant differences; p <0.05; Latent mastitis[1] – normal milk consistency, but infection is present in samples of raw milk without changing the SCC and a negative CMT score; Subclinical mastitis[2] – no symptomps are observed, the udder and milk appear normal, but infection is still present with positive CMT score and increased SCC; Clinical mastitis[3] – signs range from mild to severe with a positive CMT score, high level of SCC, positive bacteriological cultivation, changing the consistency of the milk with the presence of flakes, clots or pus and reduction or loss of milk production with clinical signs.

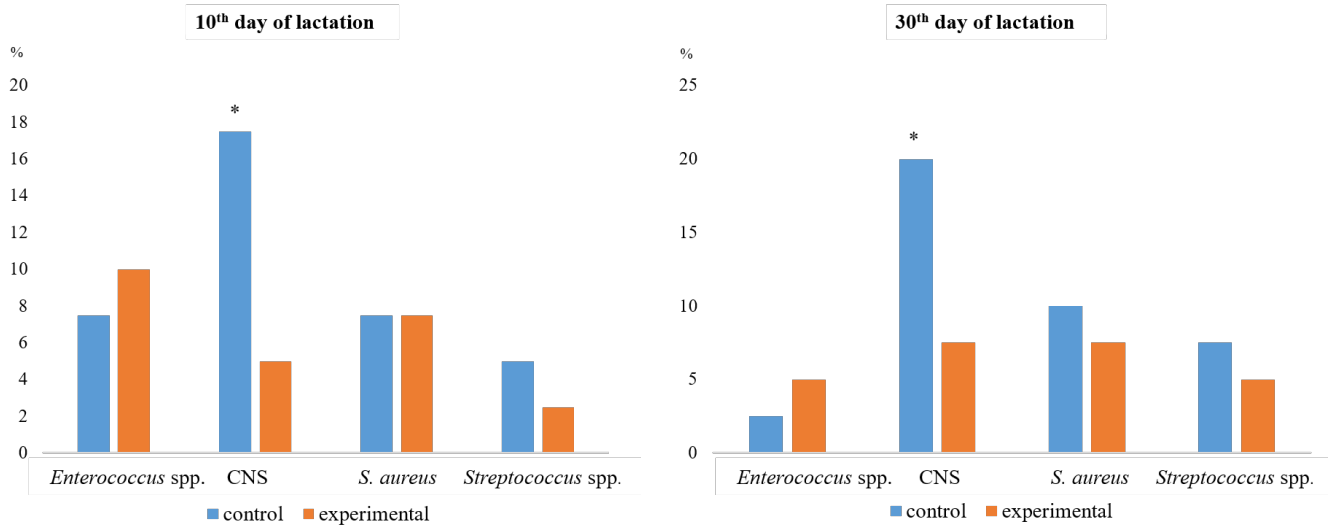

Figure 3 Distribution of bacterial pathogens causing mastitis in monitored groups (%). Note: Experimental – experimental group supplemented with HA, control – group without supplementation of HA, CNS – coagulase negative staphylococci, *Significant difference p <0.05 when significance level α = 0.05 (5%); critical value $\chi 2$= 5.99.

CONCLUSION

According to our results dietary supplementation with HA significantly reduced the MU content, SCC and the number of positive quarters detected by the CMT on the 10th day after calving but did not affect the others milk parameters. Although the mechanism by which HA supplementation affects milk synthesis and mastitis reduction has not been fully described, its indirect beneficial effects could improve the immunity of the mammary gland. Based on the information above, further research into the use of humates for the prevention of mastitis during the peripartum period and early lactation will be needed.

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POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN PSEUDOCEREALS AND THEIR PRODUCTS

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ABSTRACT
Pseudocereals are important as gluten-free crops that could be utilized as functional foods. They contain proteins with high biological value and also bioactive compounds such as phenolic compounds, flavonoids, vitamins, and minerals that can possess positive health effects on the body. Three types of pseudocereals (amaranth, buckwheat, and quinoa) were evaluated for polyphenols and antioxidant activity. Spectrophotometric methods were used for the determination of free phenols amount with Folin-Ciocalteu reagent, and total antioxidant capacity (TAC) with DPPH and ABTS reagents. Free phenols, the predominant part of polyphenols, were in pseudocereals in the range from 12.4 to 678.1 mg GAE.100g^{-1}. The highest content of FP was found in buckwheat products (146.8 – 678.1 mg GAE.100g^{-1}); quinoa and amaranth products reached much lower values (up to 226.1 mg GAE.100g^{-1}). Antioxidant activity was in an agreement with the FP amounts order, the highest TAC values were again for buckwheat products (167.3 – 473.9 and 876.9 – 3524.8 mg TE.100g^{-1}), followed by quinoa (78.2 – 100.6 and 738.9 – 984.5 mg TE.100g^{-1}) and amaranth ones (25.0 – 69.7 and 118.2 – 431.4 mg TE.100g^{-1}). A high positive correlation between FP amount and TAC values was evaluated for analyzed pseudocereals. The highest content of free phenols and the best antioxidant potential showed buckwheat wholemeal flour, so buckwheat could be characterized as a great source of free phenols with high antioxidant activity.

Keywords: pseudocereal; free phenol; antioxidant activity; DPPH; ABTS

INTRODUCTION
Pseudocereals are important gluten-free crops where belong especially amaranth, buckwheat, and quinoa. Their great nutrient properties and also suitability for the preparation of gluten-free foodstuffs **(Alvarez-Jubete et al., 2010)** predestinate them for the utilization as functional foods. They are known to have good nutritional value, specifically because of proteins with high biological value. It is due to the presence of essential amino acids (especially lysine and tryptophan) in a higher content **(Kocková and Valík, 2011)**. Due to their starch content they are also sources of energy. They contain also natural antioxidants, high levels of flavonoids (e.g. rutin, hyperoside, vitexin, isovitexin, orientin, isoorientin, catechin and epicatechin gallate in buckwheat), vitamins and minerals **(Salehi et al., 2018; Tomotake et al., 2007; Kiprovski et al., 2015)**.

Amaranth (*Amaranthus* spp.) is a rich source of proteins, with well-balanced amino acid composition and good bioavailability. It has higher lysine content than other cereal grains **(López et al., 2019; Tovar-Pérez, Lugo-Radillo and Aguilera-Aguirre, 2019)**. Amaranth is known also due to some potential health benefits (decreasing plasma cholesterol levels, reducing blood glucose levels and anemia) that have been conducted in experimental animal models **(Caselato-Sousa and Amaya-Farfán, 2012)**. Its seeds contain a good amount of polyphenols such as flavonoids with quite high antioxidant activity **(Vollmannová et al., 2013)**. To the important phenolics, there belong caffeic acid, ferulic acid and *p*-hydroxybenzoic acid **(Klimczak, Małecka and Pachołek, 2002)**.

One of the most important pseudocereal sources for functional foods is common buckwheat (*Fagopyrum esculentum* Moench). To the functional substances in buckwheat belong flavonoids, phytosterols, fagopyrins, fagopyritols, phenolic compounds, resistant starch, dietary fiber, lignans, vitamins, minerals and antioxidants **(Ahmed et al., 2014)**. Middling and bran buckwheat flours could be used to develop functional foods due to phenolic compounds presence. Phenolics are present there in the free and bound form. They are concentrated mainly in the outer layer (hull and bran) as the hull is removed before the milling of the buckwheat **(Martín-García et al., 2019)**. The study of **Li et al. (2013)** showed that rather than buckwheat flours, hulls and brans are a better source

of antioxidants. The health-promoting properties of buckwheat are expressed due to the content of antioxidants such as phenolic acids, rutin (quercetin-3-rutinoside), and fagopyrin, and specific proteins **(Ölschläger et al., 2008; Sytar et al., 2016)**. To the other health benefits belong, similarly as for amaranth, plasma cholesterol level reduction, antidiabetic properties, and also anti-inflammatory effect and improvement of hypertension conditions **(Giménez-Bastida and Zieliński, 2015)**.

Quinoa (*Chenopodium quinoa* Willd.) is a plant of the *Chenopodiaceae* family. It is also a gluten-free crop that is suitable for coeliac patients because it contains very little or no prolamin **(Jancurová, Minarovičová and Dandár, 2009)**. It is exceptionally high in lysine that is not overly abundant in the vegetable kingdom. Quinoa seeds contain also phytohormones that have a good impact on human nutrition **(Vega-Gálvez et al., 2010)**.

Processing of crops (procedures, extraction methods, used temperature, type of present compounds) can modify the polyphenol content of foods in several ways **(Manach et al., 2004; Inglett et al., 2011)**.

This study aimed to assess differences in antioxidants of pseudocereals, concretely amaranth, buckwheat and quinoa, by comparison of free phenols content and total antioxidant capacity.

Scientific hypothesis

The scientific hypothesis of this study was to examine the differences and relations between free phenolic content and antioxidant capacity measured by two methods (DPPH with IC50, and ABTS tests) in three types of pseudocereals (amaranth, buckwheat, and quinoa), and also differences between samples themselves.

MATERIAL AND METHODOLOGY

Pseudocereal samples

Three types of pseudocereals and their products (13 samples), bought from food markets of different origin, were analyzed. There were amaranth, buckwheat, and quinoa. Amaranth (4 samples of Indian, Hungarian, Czech and German origin; grains (AG), flour (AF), wholemeal flour (AWF) and particles (AP)); buckwheat (6 samples of Poland, Czech and Latvia origin; grains (BG1, BG2), flour (BF1, BF2), wholemeal flour (BWF) and groats (BGR)); and quinoa (3 samples of Peruvian and Bolivian origin; different types of grains (white QGW, red QGR, black QGB)).

Determination of Free Phenolic Content

For the determination of free phenolics content (FP) in the pseudocereals modified spectrophotometric method with Folin-Ciocalteau reagent **(Vollmannová et al., 2013)** was used.

The extracts of pseudocereal samples were prepared from 1 g of homogenized pseudocereal sample and 25 mL of methanol (80% (*v/v*); Penta Chemicals, CZ) with stirring in a shaker for 8 h at room temperature. The extract was afterward filtered through a paper filter and used for the analyses of FP.

To extracts (1 mL) with 5 mL of distilled water, Folin-Ciocalteau reagent (2.5 mL; Penta Chemicals, CZ), was added and after agitation, it was left for 3 min in the dark at room temperature. Then sodium carbonate solution (7.5 mL, 20% (*w/v*); Penta Chemicals, CZ) was added to a mixture and mixed again. The content was then filled up to 50 mL. After 2 h of the extract standing in the dark at room temperature the absorbance of samples was measured at wavelength 765 nm (Libra S6 Biochrom spectrometer, GB) against blank. As a standard a gallic acid was used. FP values were expressed as gallic acid equivalents (GAE), $mg.100g^{-1}$ sample. Determinations were made in triplicate.

Determination of Antioxidant Activity

Antioxidant activity of pseudocereals was assessed as a total antioxidant capacity (TAC). It was evaluated by a modified spectrometric method with DPPH reagent **(Vollmannová et al., 2013)** and also with ABTS reagent **(Škrovánková et al., 2018)**.

For both determinations there was used the same extraction process for analyzed samples. 1 g of homogenized pseudocereal sample was mixed with 25 mL methanol (80% (*v/v*); Penta Chemicals, CZ) with stirring in a shaker for 8 h at room temperature. The extract was afterward filtered through a paper filter and used for the analyses.

DPPH method: To pseudocereal extract (0.1 mL) a DPPH (1,1-diphenyl-2-picrylhydrazyl) solution in methanol (3.9 mL; 1 mM; Sigma Aldrich, CZ) was added. The mixture was shaken vigorously on a Vortex mixer in capped glass and left in the dark for 10 min. (room temperature). The absorbance of samples (A) and absorbance of control samples (AC) was measured on the spectrometer (Libra S6 Biochrom, GB) at λ = 515 nm against a blank. The pseudocereal inactivations (I) were calculated from the decrease of absorbance (%) according to relation (1) and the results were then expressed, using the calibration curve of standard (trolox), as trolox equivalents (TE) in $mg.100g^{-1}$ sample. Average results were obtained from three parallel determinations.

$$I = \frac{AC-A}{AC} . 100 \quad (1)$$

IC50 method: For the determination of 50% antioxidant inactivation, to scavenge 50% of DPPH free radicals, the most effective pseudocereal type, buckwheat, was used. For IC50 method there were prepared five diluted methanolic buckwheat extract solutions for each sample, in the range 1 – 10 $mg.mL^{-1}$. The reaction mixtures were made the same way as for TAC (DPPH) determination. From the results of inactivation for these extract concentrations the IC50 values were quantified by linear regression.

ABTS method: To 50 µL of pseudocereal extracts 4 mL of the reactive radical mixture composed of ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; Sigma Aldrich, CZ) (12 mL; 3.5 mM) with $K_2S_2O_8$ (0.06 M; Lukes, CZ) and acetic buffer (pH 4.3), was added. The solution was shaken vigorously on a Vortex mixer and left to react without light exposure for 30 min at room temperature. Pseudocereal samples absorbance (A) and absorbance of control samples (AC) were then measured by a spectrometer (Libra S6 Biochrom, GB) at wavelength 734 nm against a blank. Inactivations (I) were

calculated from the decrease of absorbance (%) according to relation (1). Results of TAC (ABTS) were calculated from inactivation using a calibration curve with trolox as a standard. It was expressed as trolox equivalents (TE) in the mg.100g^{-1} sample. Average results were obtained from three parallel determinations.

Statistical analysis

All data were expressed as mean values ± standard deviation (SD), every determination was made in triplicate. Statistical analysis of the results was made by Statistica program, StatSoft version 9.0 (Dell, USA) using parametric test comparing mean values of two independent assortments (Student *t*-test). Differences at a 95% confidence level (p <0.05) were considered statistically significant. Correlations between the evaluated parameters were obtained using Pearson's correlation coefficient (r).

RESULTS AND DISCUSSION

Content of phenolics

Antioxidants that could donate electrons, such as polyphenols, vitamin C (ascorbic acid), and vitamin E (tocopherols), were evaluated by the method with Folin-Ciocalteau reagent.

As **Li et al. (2013)** found out predominant polyphenols in pseudocereals, such as buckwheat, are free phenolics (FP), accounted for about 94.07% of whole polyphenol content.

The amounts of FP in the pseudocereal samples in our study (Table 1) ranged from 12.4 to 678.1 mg GAE.100g^{-1} with the average 208 mg GAE.100g^{-1} pseudocereal sample. There were marked differences between individual pseudocereals (p <0.05, Student *t*-test).

The highest content of FP was analyzed for the buckwheat products; quinoa and amaranth products reached much lower values. These findings are in agreement with **Alvarez-Jubete et al. (2010)** results for pseudocereal seeds determination. Also **Vollmannová et al. (2013)** and **Gorinstein et al. (2008)** detected for pseudocereals this order of polyphenols amount.

The average FP value for buckwheat seeds and products in our research was 357 mg GAE.100g^{-1}, that is up to 3 times higher than in quinoa (average 141 mg GAE.100g^{-1}) and up to 10 times than in amaranth (average 34 mg GAE.100g^{-1}) seeds and their products. The highest amount was analyzed for buckwheat wholemeal flour followed by other types of buckwheat flours.

In **Alvarez-Jubete et al. (2010)** study polyphenol amount for buckwheat seeds was 323 mg GAE.100g^{-1}, for amaranth 21.2 and for quinoa it was 71.7 mg GAE.100g^{-1}. Buckwheat values of our determination were similar also to the contents of **Salehi et al. (2018)** study that were in the range from 265 to 430 mg of caffeic acid equivalents per 100g.

In a study of **Gorinstein et al. (2007)**, the total phenol content in amaranth and quinoa reached similar values (40.5 – 43 and 91.2 mg GAE.100g^{-1}, respectively) as in our study, for buckwheat it was much less, 60 mg GAE.100g^{-1}. **Vollmannová et al. (2013)**, however, found in seeds of selected pseudocereals a higher amount of phenolics than evaluated in our study for buckwheat; quinoa, and amaranth seeds and their products.

Antioxidant activity

To evaluate the antioxidant potential, the antioxidant activity of all selected pseudocereal seeds and their products was measured. Two methods, DPPH and ABTS tests were used. The results of total antioxidant capacity (TAC) are summarized in Table 1.

The TAC values for the DPPH method were in the range from 25 to 473.9 of trolox equivalents per 100 grams of pseudocereal sample with an average 166 mg TE.100g^{-1}. Results of ABTS test were from 118.2 to 3524.8 mg TE.100g^{-1}, the average 1280 mg TE.100g^{-1}. For the results of both methods there were marked differences between pseudocereals (p <0.05, Student *t*-test), similarly as for free phenols.

From the evaluation of the results it can be seen that the pseudocereal type with higher antioxidant values is buckwheat, followed by quinoa and amaranth. It is the same order as for FP values. These findings are in agreement with the values previously reported by **Alvarez-Jubete et al. (2010)** for pseudocereal seeds. The studies of **Gorinstein et al. (2007)**, **Gorinstein et al. (2008)** and **Vollmannová et al. (2013)** introduced for pseudocereals the same order for antioxidant capacity results.

Buckwheat samples for DPPH and ABTS test in our study reached 2 – 19 and 2 – 30 times higher values than in quinoa samples (average 92 and 860 mg TE.100g^{-1}, respectively) and 2 – 6 and up to 5 times, respectively, than in amaranth (average 41 and 263 mg TE.100g^{-1}) seeds and their products. As for particular samples, the best product with the highest antioxidant activity, measured by both methods, was buckwheat wholemeal flour followed by other types of buckwheat flours and buckwheat grain (BG1).

A similar trend of buckwheat samples is shown for IC50 values in Figure 1. IC50 expresses the concentration of buckwheat extracts requisite for 50% inhibition, therefore the highest antioxidant capacity is showed by the lowest IC50 value. IC50 concentrations of buckwheat extracts were in the range 2.391 – 6.520 mg.mL^{-1}, the average 4.218 mg.mL^{-1}. Wholemeal flour (BWF) had the lowest IC50, nearly 3 times lower than the sample with the highest IC50 value (buckwheat grain BG2). So wholemeal flour has the highest antioxidant activity what could be seen also from DPPH and ABTS tests.

Alvarez-Jubete et al. (2010) reported analogous order of antioxidant capacity for pseudocereal seeds, determined by DPPH assay, the value of 620 mg TE.100g^{-1} for buckwheat, 57.7 for quinoa, and 28.4 mg TE.100g^{-1} for amaranth, respectively. In the study of **Salehi et al. (2018)** TAC results (DPPH test) in buckwheat seed samples varied from 268 to 628 mg TE.100g^{-1}. **Zielińska et al. (2012)** determined TAC in buckwheat seeds 215 mg TE.100g^{-1}.

Despite some variations in exact values of antioxidant potential in our research and other studies, they are comparable, and buckwheat could be reported as the greatest source of polyphenols with the highest antioxidant activity amongst pseudocereals and also some other cereals too **(Gorinstein et al., 2007; Gorinstein et al., 2008; Gallardo, Jiménez and García-Conesa, 2006; Zieliński and Kozlowska, 2000)**.

Table 1 The content of free phenols (FP) and values of total antioxidant capacity (TAC) in pseudocereals.

Pseudocereal sample	FP (mg GAE.100g^{-1} ±SD)	TAC (DPPH) (mg TE.100g^{-1} ±SD)	TAC (ABTS) (mg TE.100g^{-1} ±SD)
AG	12.4 ±0.7^{a}	26.4 ±1.3^{a}	118.2 ±8.3^{a}
AF	31.5 ±1.2^{a}	25.0 ±0.9^{a}	173.0 ±13.8^{b}
AWF	19.2 ±1.6^{a}	69.7 ±1.8^{b}	431.4 ±25.0^{c}
AP	71.6 ±3.5^{c}	44.2 ±3.5^{c}	327.9 ±17.9^{d}
BG1	292.5 ±10.8^{d}	280.0 ±11.5^{d}	2031.6 ±78.7^{e}
BG2	146.8 ±3.2^{e}	167.3 ±5.1^{e}	876.9 ±65.2^{f}
BF1	347.2 ±16.9^{f}	266.1 ±13.9^{f}	2287.4 ±90.4^{g}
BF2	354.9 ±18.0^{f}	291.6 ±14.2^{d}	2795.7 ±81.2^{h}
BWF	678.1 ±21.3^{g}	473.9 ±22.4^{g}	3524.8 ±121.6^{i}
BGR	321.4 ±10.1^{f}	242.0 ±9.1^{h}	1528.3 ±60.8^{j}
QGW	226.1 ±9.4^{d}	100.6 ±7.7^{i}	984.5 ±51.3^{k}
QGR	97.3 ±4.8^{h}	78.2 ±6.2^{b}	826.1 ±41.8^{f}
QGB	100.5 ±5.6^{h}	97.4 ±2.8^{i}	738.9 ±37.2^{l}

Note: Means within a column with at least one identical superscript are not significantly different by Student's t-test (p <0.05).

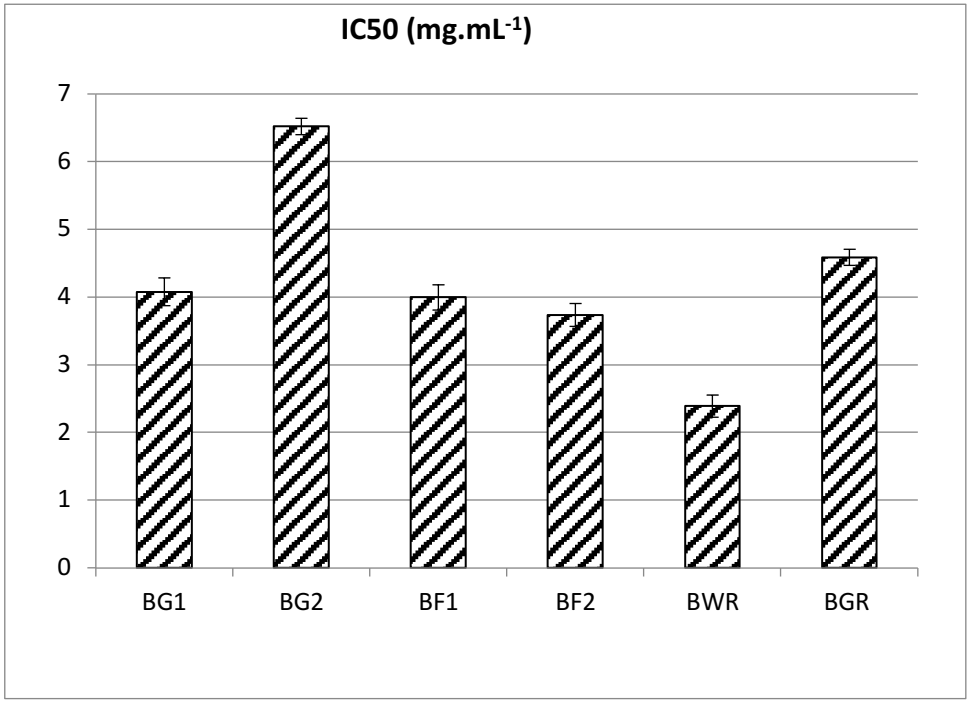

Figure 1 IC50 values (DPPH test) (mg.mL^{-1}) of buckwheat samples.

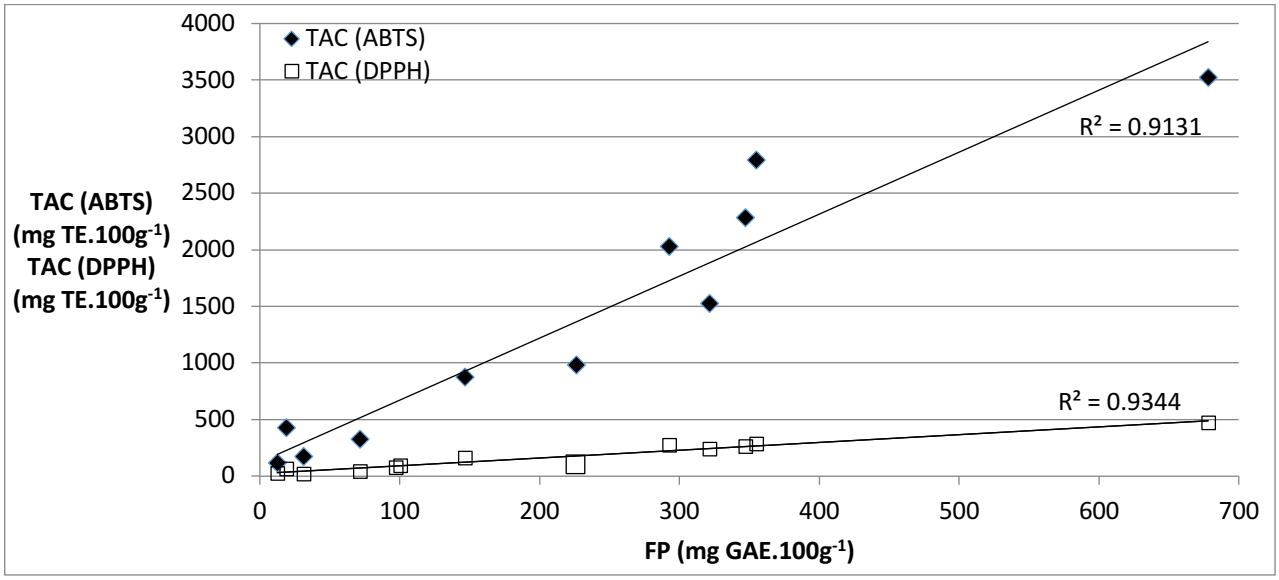

Figure 2 Correlations between FP and TAC values (DPPH, ABTS tests) of pseudocereals.

Pseudocereal samples exhibited similar order of samples for FP and TAC values (DPPH and ABTS assays). The relationships between them are shown by the correlations in Figure 2. They are strongly related to a correlation factors $r = 0.9666$ and 0.9565, respectively. In the research of **Sun and Ho (2005)** there was also found a significant correlation (0.96) between polyphenols content and antioxidant activity (DPPH method), in buckwheat extract. In amaranth and quinoa extracts there were reported weak correlations between polyphenols content and antioxidant activity by **Nsimba, Kikuzaki and Konishi (2008)** study.

CONCLUSION

Pseudocereals contain bioactive compounds such as phenolic compounds, flavonoids that can possess positive health effects on the body.

Amaranth, buckwheat, and quinoa were evaluated by spectrophotometric methods for the determination of free phenols amount and total antioxidant capacity. Free phenols in pseudocereals were in the range from 12.4 to 678.1 mg GAE.100g^{-1}. The highest contents of FP were found in buckwheat products; quinoa and amaranth products reached much lower values (up to 226.1 mg GAE.100g^{-1}). Evaluated antioxidant activity, the highest TAC values were determined again for buckwheat products (up to 473.9 (DPPH test) and 3524.8 (ABTS test) mg TE.100g^{-1}), followed by quinoa (up to 100.6 and 984.5 mg TE.100g^{-1}, respectively) and amaranth ones (up to 69.7 and 431.4 mg TE.100g^{-1}, respectively). Antioxidant capacity values by two evaluation methods (DPPH, ABTS) are in agreement with polyphenols content order. The highest content of free phenols, and also the best antioxidant potential, showed buckwheat wholemeal flour. Our study is generally in agreement with the findings of previously reported researches focused on pseudocereals. Buckwheat therefore could be characterized as a great source of free phenols with high antioxidant activity and thus could be used as seed for production of high nutritional quality products, especially for people who do not could eat cereal products due to the gluten presence. Buckwheat seeds could be also added to other cereal products to heighten their nutritional quality.

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INFLUENCE OF PACKAGING ATTRIBUTES ON PERCEPTION OF JUICE: EYE-TRACKING STUDY

Ján Nemergut, Stanislav Mokrý

ABSTRACT

Today, consumers are increasingly aware of the impact that the fast and stressful way of life has on their health. They focus not only on physical activity, but also on a diet filled with fruits and vegetables. As a result, they often choose a tasty alternative which is one of the main sources of vitamins and nutrients - fruit juices. However, these products are often labeled as drinks with high amounts of sugar. Therefore, it is very important for these drinks to be perceived by the consumers as healthy and tasty, which is one of the most important features of their packages. Their goal is to appeal to customers, catch their attention and make them buy the product. One of the most convenient methods to study how packages appeal to customers is the eye-tracking method. The aim of this article is to find out how different attributes of packages can influence customers' perception of the juice. The research was carried out in a form of eye-tracking experiment (A/B testing), which involved 38 participants at the age from 20 to 29 (generation Y). Results showed that lower color saturation significantly reduces the attention of individual packages and also reduces the influence of craving the juice as opposed to brighter colors. The importance of information on the back side was also confirmed, since moving the information from back to the front side did not show any significant decrease of the back side's attention span. Last but not least, it has been found out that the image type used on the orange juice package holds importance too, since photography of oranges led to a higher craving of the juice in comparison to the illustration of oranges. However, it was not proven that photographs of oranges held a higher attention span compared to the illustrations. The article contains demonstrable proof of individual package attributes' influence on how generation Y consumers perceive the juice.

Keywords: consumer; attribute; juice; eye-tracking; package

INTRODUCTION

In recent years, the consumers' attention is focusing on a healthy lifestyle, which is also related to a healthy diet. There is a permanent trend among global and state institutions which recommend higher consumption of fruit and vegetable due to positive effects on the organism. This is one of the reasons why the vast majority of consumers perceive juices as beneficial for health **(Nicoli, 2012)**. Their popularity confirms the fact that while in the past, they were created as a result of the overproduction of fresh fruit, nowadays the fruit (mostly citruses and apples) is in many areas grown specifically for juicing **(Robertson, 2009)**. Fruit juices contain mainly vitamins and minerals but also saccharides prevailing in the solid component. Even though the fruit contains only a small amount of proteins and fats, there are higher concentrations of iron, calcium, and vitamins B and C **(Ashurst, 2016)**.

The area of drinks, and more specifically juices, belongs to a group of products that are bought impulsively (habitually) by consumers **(Mruk-Tomczak, Jerzyk and Wawrzynkiewicz, 2019)**. This means that during the decision process, they create little to no conscious effort, they don't search for information beforehand and as a result, they're often tempted to buy impulsively **(Solomon, 2009)**. The product's packaging design plays a crucial role in this form of shopping **(Cahyorini and Rusfian, 2012; Hubert et al., 2013; Waheed, Khan and Ahmad, 2018)**.

In recent years, there have been countless researches about the influence on the decision making of consumers during shopping for groceries. One of the researched attributes was the shape and overall package design. According to **Burgess (2016)**, the shape of a specific product package is very important for the consumer, since people are visually driven beings and therefore the color and overall package design have a much bigger influence on expectations and consequential experience of the consumer compared to holding the product itself in their hands as such. Nowadays, there is a lot of research carried out on the so called "crossmodal correspondence". It stands for cross sense associations, e.g. between letter shapes and taste **(Velasco**

et al., 2014), package shape and perceived health safety **(Fenko, Lotterman and Galetzka, 2016; Ooijen et al., 2017)**, smell and shape **(Hanson-Vaux, Crisinel and Spence, 2012),** etc. where consumers imagine a certain taste, smell and so on based on a certain shape. The importance of packaging colors has also been proven within impulsive shopping **(Bone and France, 2001)**. The research focused on color harmony **(Wei et al., 2014; Hurley et al., 2017)**, color hues **(Ampuero and Vila, 2006)**, but also the influence of colors on product's taste itself **(Piqueras-Fiszman and Spence, 2011; Karnal et al., 2016; Rompay, Deterink and Fenko, 2016)**. Within the products, consumers are now also focusing on the health side of the products, and therefore the impact of the information on the perception of the health acceptance of the product is being examined increasingly. On that account, the subject of the research was, for example, the presentation of health claims on the packages **(Liem, Aydin and Zandstra, 2012; Sütterlin and Siegrist, 2015)**, the presentation of nutrition claims **(Talati et al., 2017)** or the impact of images on the packaging on consumer's perception **(Mizutani et al., 2010)**.

It is necessary to realize that these researches are not only a mere theory, but it is indeed possible to implement gained conclusions in practice. As one of the examples may serve the redesign of the orange juice package of one of the world's largest fruit juice processors, Tropicana. When changing the package of Tropicana's pure orange juice, the typical feature of the juice packaging - an orange with a straw - was removed. This has made it impossible for consumers to find this particular feature, which means that they cannot identify the new package and, of course, do not buy it. The new juice packaging resembled a private brand. The change in the company's packaging resulted in a significant drop in sales by 20% between December 2008 and February 2009, which is an estimated loss of 27.3 million dollars. The redesign of the new packaging cost Tropicana about 35 million dollars and is considered to be the most important event in the beverage industry **(Lee, Gao and Brown, 2010)**.

Many other researches have been, are and will continue to be conducted within the design of packages, to reveal some links between packaging attributes and consumer's perception. The goal of this study is to test certain assumptions of the impact of packaging on the perception of Y-generation consumers in the field of fruit juices by using eye-tracking technology and thereby also contributing to further progress in this field of research.

Scientific hypothesis

H_0: Color saturation of juice package has no impact on the package's attention span.

This hypothesis is based on the assumption that the higher the package color saturation is, the higher level of attractiveness and expectations for its consumers it has **(Velasco and Spence, 2018)**.

H_0: Additional information on the front side of the juice package has no impact on the inspection duration of the back side of the juice package.

This hypothesis is formulated based on the general assumption that the consumers will spend less time looking at the back side of the juice package after moving the information from the back side to the front side.

H_0: Type of package image has no impact on the length of observation of the juice package.

This hypothesis includes the assumption that consumers prefer photography of a certain object over its illustration **(Kovač et al., 2019)**, therefore they should spend more time looking at the package with photography compared to the package with an illustration.

MATERIAL AND METHODOLOGY

The research consisted of two parts, where the first part was eye-tracking research and the second part was an in-depth interview. The research was held from the 14th to 18th of October 2019 in eye-tracking laboratory ETLab of the department of economics at Mendel University in Brno. The respondents applied for this research via Google Docs application shared through social media Facebook. Requirements for participation in the research were age between 20 to 30 years and consumption of fruit juices. 38 respondents have participated in the research, during which none of the respondents experienced loss of signal or incorrect calibration, which could significantly influence the eye-tracking research. Half of the participants were in the age between 20 to 24, the other half belonged to the age group between 25 to 29. The average age of the participants was 24.53 years. 58% of the respondents consisted of women (22) and the remaining 42% of men (16). The largest representation consisted of Slovak nationality (25), which is roughly 65%. The second largest representation was one of Czech nationality (12), which is roughly 33%. One of the participants was of Ukrainian nationality and has been living in the Czech Republic for the last 5 years and speaks fluent Czech. Table 1 shows the estimated monthly juice consumption in liters among individual respondents.

Table 1 Estimated monthly juice consumption in liters among individual respondents.

1 – 2 L	15 respondents	39.47%
2 – 5 L	16 respondents	42.10%
5 – 7 L	6 respondents	15.80%
7 – 10 L	1 respondent	2.63%
More than 10 L	0 respondent	0%

The research was in a form of experiment and A/B testing, where 19 respondents in group A were presented with one variant of stimuli, and the other 19 respondents in group B were presented with another variant of stimuli with variations. This research used a static eye-tracker SMI RED 250 produced by SensoMotoric Instruments GmbH (hereinafter referred to as SMI), which was placed under screen's monitor with resolution 1680×1050 px. Eye-tracking reading was carried out on a frequency of 120 Hz. The software used for eye-tracking research was Experiment center SMI, as well as SMI iView X, which was used to control the eye-tracking. First, a 5-point calibration was performed, followed by validation, which provided information about the deviation from the calibration point. For this thesis, a maximum deviation of 0.5 degrees was determined on both the x-axis and y-axis. Calibration and validation were performed multiple times while choosing the best value. The eye-tracking research consisted of several blocks. At the beginning of each block, instructions were given, after which the respondents continued by pressing the spacebar on a keyboard. After

each task description (before displaying each stimulus), a so-called "fixation cross" was displayed for 800 ms. The goal of this cross was to direct the participant's eyes to the center of the screen, therefore allowing each stimulus to be viewed from the same place. All stimuli (juices) were displayed in the background with RGB color: 192, 192, 192. Respondents answered a specific question each time an image was displayed. Respondents could view each stimulus indefinitely and press the spacebar to continue to the next image. After the eye-tracking research, an in-depth interview took place, where the participants were asked several questions regarding the eye-tracking, as well as general questions about the consumption of juices.

For the processing of the eye-tracking research data, SMI BeGaze software was used to create individual areas of interest (AOI). These were the individual parts of the packaging that, in certain cases, differed in groups A and B and were the subjects of research. These areas of interest contained metrics also known as key performance indicators (KPIs). These KPIs may for example indicate how many participants viewed each element, in which order and with how many repetitions. Based on the data, heat maps were also created, where red indicates the areas with the most attention, while blue indicates the least attention.

The hypotheses were tested using paired *t*-test and Mann-Whitney test using IBM SPSS Statistics 25 statistical software.

Stimuli

The research used pictures of fruit juice packages of foreign brands, but also of juice brands sold in Czech and Slovak markets (Figure 1). In the case of the first hypothesis, which concerns the effect of the color saturation, the color saturation of each alternative was reduced by 50%. In the second stimulus, the information from the back side of the juices was added to the front side of the juices in a form of leaves that fitted the entire package design better. In the case of the third hypothesis, packages with illustrations of oranges were created in addition to the juices with images of real oranges. The effect of the image type was observed in the case of the packages, where the image was a small part of the package, as well as in the case where it created a larger part of the package. All graphic edits were done with Adobe Photoshop CC2018 software.

RESULTS AND DISCUSSION

The first stimulus consisted of a selection of 4 juices, 2 of which were colored normally and the remaining two had their color saturation reduced by 50%. Based on the data of individual metric, it has been recorded that the color saturation had an impact on the number of returns to a certain juice. The largest difference has been recorded in juice brand Tropicana, where the respondents looked back 3 times at the version with lower color saturation, while the normally colored version caught their attention more than 5 times. In case of juices Minute Maid and Tropicana, the most observed area of interest was the brand, while for the remaining two juices Del Monte and Dimes it was the flavor labeled 100%.

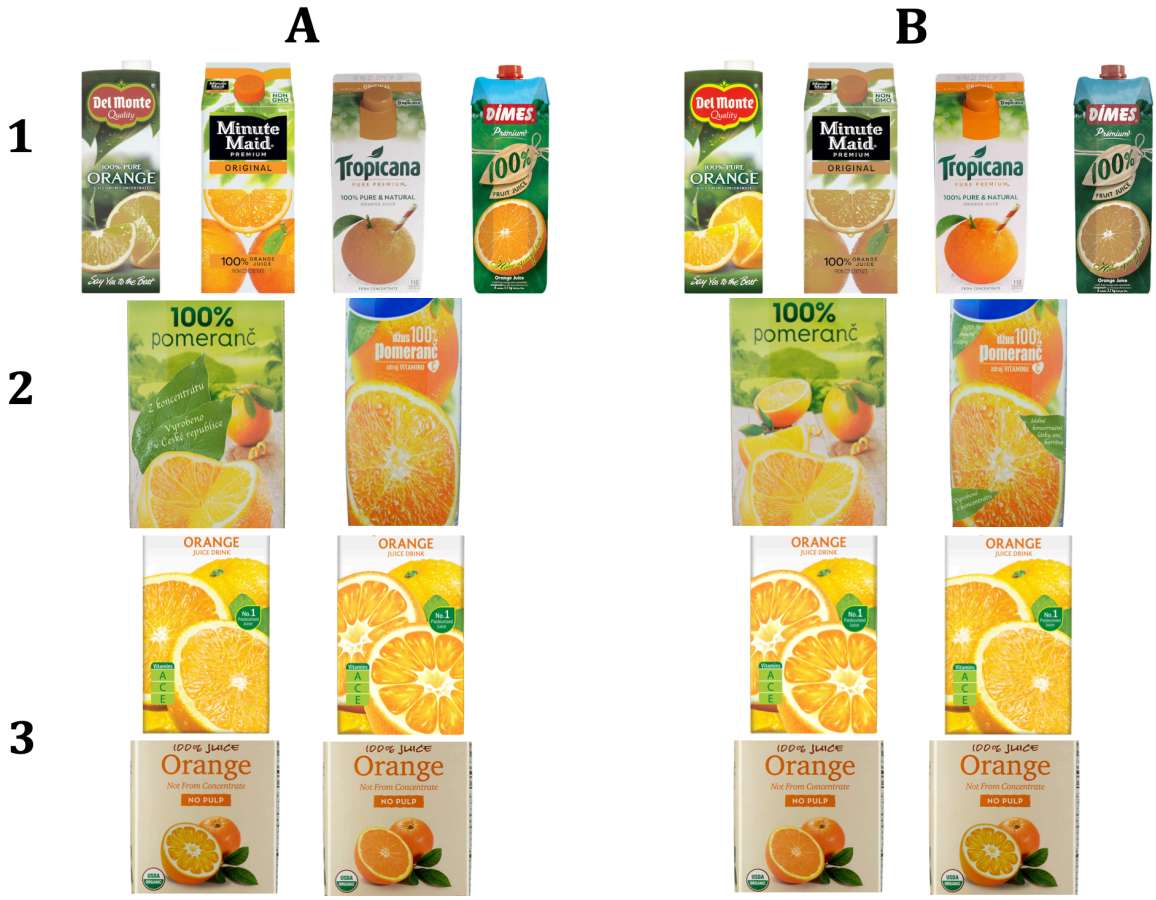

Figure 1 Variants of stimuli used for A/B test.

Table 2 Average dwell time of selected AOIs of juices from the first part of eye-tracking research.

		Normal saturation	Lower saturation
Del Monte	Brand	4.1%	4.9%
	Picture	5.9%	4.3%
	Flavor + 100%	9.3%	5.0%
Minute Maid	Brand	20.0%	13.1%
	Picture	5.7%	4.6%
	Flavor + 100%	3.1%	1.8%
Tropicana	Brand	10.4%	7.4%
	Picture	6.0%	2.5%
	Flavor + 100%	3.1%	2.0%
Dimes	Brand	4.2%	1.7%
	Picture	4.9%	4.1%
	Flavor + 100%	5.9%	6.6%

Table 2 shows the average dwell time in the percentage of selected areas of interest at lower and higher color intensities. Based on these numbers we're able to conclude that higher saturation positively impacts the attention paid to the selected areas of interest. This impact of saturation has been statistically tested with the first hypothesis, which has been observed individually at each juice. Table 5 shows that the *p*-value of tests is lower than the significance value $\alpha = 0.05$ in three out of four tests, therefore the null hypothesis is rejected and the alternative hypothesis is accepted instead. Therefore, it can be concluded that most of the offered juices showed a statistically significant effect of color saturation on the specific juice's attention. In the case of the juice Del Monte where the null hypothesis of the nonexistence of color saturation impact on dwell time was not rejected, Table 5 shows higher average dwell time in the case of higher color saturation. We are therefore able to observe the positive impact of color saturation in this juice, even though it has not been confirmed as statistically significant. **Kovač et. al (2019)** also observed the same impact of brighter colors during his research, which confirmed that brighter colors are more attractive to consumers. Brighter colors not only impacted the dwell time of consumers but also their selection of the juice. During the eye-tracking research, they were asked to choose one of the four juices they fancied the most. Within group A, it was very clear since 18 respondents (95%) chose juice Minute Maid, while 1 respondent (5%) chose juice brand Dimes. Both brands had fully colored packaging. Group B was less clear. Juice Minute Maid was fancied the most by 4 participants (21%) despite the lowered color brightness in packaging. The remaining participants would choose juices with higher package color saturation, more specifically 5 (26%) would choose Del Monte and 10 respondents (53%) would choose juice Tropicana. We can conclude that the packaging colors are being subconsciously perceived by consumers who may be influenced by more saturated colors in the form of fancying the juice more. This fact has also been clarified in a study by **Wei et al. (2014)** who explains that saturated colors create a new expectation of a fresh fruit product in consumers. Juice package with bright colors may create an impression of a freshly squeezed juice. The higher presence of orange color on juice packages Tropicana and Minute Maid may have caused higher dwell time with these brands. **Wei et al. (2014)** and **Ježovičová, Turčinková and Drexler (2016)** state that it is appropriate to use a color in the background of the juice package that corresponds to the fruit from which the juice is prepared. **Schloss and Palmer (2010)** found in their research that the evaluation of color preferences and harmony was highest for colors that have the most similar hues. This could also have resulted in a successful selection of these juices by the respondents. The mentioned fact may have also contributed to the choosing of these juices by the respondents. On the other hand, **Hurley et al. (2017)** did not confirm the significance of color harmony on package preference in his study. He stated that designers have creative freedom in package design and are not required to follow this rule.

In the second part, we researched whether an edit of the front side of juice packaging can impact the reviewing process of the juice by the respondent. Two Czech juice brands Hello and Relax in tetrapack package were chosen as the stimuli. The front sides of the packages were edited with additional information from the back sides in the form of texts placed in leaves. In the case of the juice Hello, the added information were "z koncentrátu" (from concentrate) and "Vyrobeno v České republice" (Made in Czech Republic). In case of the juice Relax, the added information was "100% obsah ovocné složky" (100% fruit content), "Žádné konzervační látky ani barviva" (No preservatives, no artificial colors) and "Vyrobené z koncentrátu" (Made from concentrate). The choice of this information was made regarding the study results by **Gadioli et al. (2013), Mohebalian, Cernusca and Aguilar (2012)** and **Romano, Rosenthal and Deliza (2015)**.

Based on the heat maps in Figure 2, it is clear that the graphic edit of the front side of the packaging drew significant attention from the respondents. Figure 2 also shows that attention paid to additional information increased at the expense of the sign "100% pomeranč" (100% orange) and the image of an orange. The most viewed part of the front side was leaves with information (37.5%), while with the original package without any edits it was the brand name (21.9%). Great attention while viewing the front side of the package was also paid to juice flavor along with the 100% sign, which the respondents in

Figure 2 Heat maps of tested variants juice Hello with and without additional informations.

group A paid attention to for about 16% of viewing time, while in group B they spent about a quarter of it.

However, the method of viewing the back side of the juices was the same in both variants. In both groups, participants spent more than 50% of the time reading the juice composition in the Czech and Slovak languages. Only 2% of viewing time was spent on each of the other areas of interest such as producer, volume, producer's website label, etc.

Similar to the Hello juice, the Relax brand can be seen in Figure 3, where we can observe the attention of the respondents with accompanied information on the front side. With the front page without additional information, the areas of greatest attention are flavor along with 100% (46.3%), brand (24.9%) and orange image (14.4%). In case of the front page with information, the flavor along with 100% (30.3%) and brand (19.7%) are also the main areas of interest but are followed by "no preservatives or artificial colors" (14.3%) and "100% fruit content" (9.5%). Only then follows the orange picture (4.6%). The label "made from concentrate" received less than 3% attention. This lesser attention could also be due to the position of this label, as it was found out that consumers prefer the upper part of the package **(Juravle et al., 2015; Rebollar et al., 2015)**.

Even in the case of the back of the Relax Juice, the most observed part was the juice composition, at which participants spent up to 40% of their time. However, there were two other areas of interest in this juice, where the participants spent a certain amount of time. Those are the nutritional values, that group A respondents went through for about a quarter of the time, while in group B the participants spent a little less time there (only about 15%). The respondents' attention was also drawn to the graphic element located in the center of the package, which spoke of shaking the juice before opening. Participants in group A spent 13.9% of the time and group B spent 11.6% of the time in this area of interest.

In this case of the eye-tracking research, we tested the assumption that when placing additional information from the back of the juice package to the front, the respondents spend less time looking at the back of the juice package. The hypothesis was tested for both juices, but in neither case the *p*-value of the tests did fall below the significance level of 5% (Table 5) and therefore the null hypothesis cannot be rejected. Thus, there is no statistically significant difference between the time of observation of the back side for additional information on the front side of the juice. With Relax juice, the *p*-value (0.0597) is relatively close to the rejection of the null hypothesis. This is also shown by the average back side viewing time, which is significantly lower if the front page information is added, although this has not been confirmed statistically. From Table 5, it can also be noted that the average back side viewing time is even longer in the case of Hello juice if there is additional information on the front side in comparison to its absence. This could also be due to the number of information added to the front, which was higher in the case of Relax, but specific information could also have an impact. Indeed, Relax juice had information about vitamin C on the front and according to **Gadioli et al. (2013)**, this information is very important for young people and students. Therefore, along with additional information, the front of Relax juice could have had a more meaningful value than Hello juice.

Figure 3 Heat maps of tested variants juice Relax with and without additional informations.

However, these incentives have confirmed the importance of the back juice packaging, which is significant for consumers. This was also confirmed in an in-depth interview where up to 32 (84%) of respondents said they always or frequently read the back of juice containers while the remaining 6 (16%) do not read this information.

Recent packages of fruit juices in this research have been examined for the perception of different types of presented oranges. For each pair of juices, the respondents were to choose the one that makes them fancy orange juice the most.

For the first stimulus, juice 365 Everyday Value, it is clear from the heat maps (see Figure 4) that respondents were primarily driven by orange images during the decision process. This is also confirmed by the authors **Simmonds and Spence (2017)**, who confirms that food images can evoke hunger or craving for food in the consumers' minds. The average percentage values of dwell time for individual areas of interest are shown in Table 3, which shows that the participants spent most of their time looking at pictures of oranges on the juice packaging. They spent up to 30% of their total viewing time by looking at orange images.

Figure 4 Heat maps of tested variants juice 365 Everyday Value with photo and illustration of oranges.

Table 3 Average dwell time of selected AOIs of juice 365 everyday value.

	Photo of oranges	Illustration of oranges
Opening system	1.35%	1.45%
Brand	8.45%	7.40%
Flavor + 100%	11.10%	9.25%
Picture	15.10%	16.10%
Additional information	2.40%	2.00%

In both groups, although the oranges in the form of illustrations were viewed longer, the difference was only 1%. The second most viewed part of the package was the 100% composition label along with the flavor, which is also confirmed by the heat map on which the area is colored as well. Juice brand viewing ranked third. The respondents paid the least attention to the uppermost part of the package (opening system) as well as the lowermost part of the package, where the volume and health claims were stated.

In the second stimulus, the package also differed in the form of oranges (graphic/real), but in this case the oranges covered a larger package area than the 365 Everyday Value juice. With the Peel Fresh juice, however, it is not possible to speak of a clear way of monitoring the juice package as it was with the first stimulus. From Figure 5 it can be stated that in this juice, the participants checked the package from top to bottom, which was due to a relatively small number of elements on the package. Table 4 shows the average percentage times of viewing specific areas of interest in Peel Fresh juice. In the case of both juices, the respondents spent about 50% of their total viewing time looking at oranges. With the brand, the average was about 13% of the viewing time. When comparing the juice packages (Figure 5), it is shown that some attention is also paid to the vitamin elements and the "No. 1 Pasteurised Juice" label. This could be because when comparing the appearance of oranges, the respondents looked from one package to another with the aforementioned elements, and therefore the respondents' eyes were focused on them for a certain amount of time.

Table 5 also shows the results of the last third tested the hypothesis, which examined the assumption that the photographs of oranges placed on the package will draw the

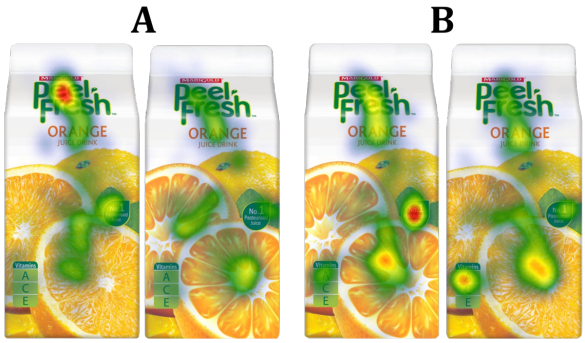

Figure 5 Heat maps of tested variants juice Peel Fresh with photo and illustration of oranges.

respondents' attention more than the illustrated oranges. However, the *p*-values of both tests did not reject the null hypothesis, thereby confirming an alternative hypothesis that suggests statistical insignificance of the image type for attention to packages. However, Table 5 shows that higher average attention was recorded in both cases of juices with a genuine depiction of orange.

Although the type of image did not have a significant effect on the length of attention, it affected fancying the orange juice. The respondents' answers about evoking the biggest appetite for juice when choosing from two versions of 365 Everyday Value juice packages clearly say that respondents prefer photographs of oranges over illustrations.

Up to 35 out of 38 respondents (92%) chose the real orange option, while only 3 participants (8%) had a greater appetite for juice when looking at the graphic version. A similar result can be observed with Peel Fresh juice.

Up to 33 participants (87%) fancied a juice package with real oranges more, while 5 respondents (13%) fancied a package with a graphic version of oranges. These results

Table 4 Average dwell time of selected AOIs of juice Peel Fresh.

	Photo of oranges	Illustration of oranges
Brand	7.30%	5.50%
Flavor	3.85%	3.85%
Picture	24.45%	25.00%

Table 5 Results of tested hypothesis.

Hypothesis	Brand of juice	Variant	Average dwell time	Test	*p*-value	Test result
1	Del Monte	Normal saturation Lower saturation	22.61% 16.39%	Independent samples Mann-Whitney	0.084930	H_0 accepted
	Minute Maid	Normal saturation Lower saturation	35.95% 24.15%	Independent samples *t*-test	0.002242	H_1 accepted
	Tropicana	Normal saturation Lower saturation	24.16% 14.84%	Independent samples Mann-Whitney	0.009766	H_1 accepted
	Dimes	Normal saturation Lower saturation	20.05% 15.24%	Independent samples *t*-test	0.032048	H_1 accepted
2	Relax	Without information With information	15651.64 ms 9446.30 ms	Independent samples Mann-Whitney	0.059692	H_0 accepted
	Hello	Without information With information	9815.09 ms 12071.76 ms	Independent samples Mann-Whitney	0.511257	H_0 accepted
3	Peel Fresh	Photo Illustration	43.79% 40.80%	Independent samples *t*-test	0.134955	H_0 accepted
	365 Everyday Value	Photo Illustration	43.77% 41.81%	Independent samples *t*-test	0.343758	H_0 accepted

are in line with the results of other studies that have shown that food product photography gives consumers feel of a healthier and more natural product **(Kovač et al., 2019; Smith, Barratt and Sørensen, 2015; Abrams, Evans and Duff, 2015; Pensasitorn, 2015)**.

This study has shown interesting results in the graphic design of juice packages, but it can also provide resources for further research. Research of the form of the fruit's presentation on the juice package proves to be suitable. The way of fruit processing has already been studied by **Machiels and Karnal (2016)**, but it would also be useful to find out what type of fruit serving produces the greatest appetite for juice (sliced, whole…).

A certain extent of limitation of this study can be seen in the unequal distribution of men and women, as the majority of the sample were women. This could have had an impact on the overall data obtained. Indeed, in some studies **(Orquin and Scholderer, 2011; Abdi Sargezeh, Tavakoli and Daliri, 2019; Sammaknejad et al., 2017)**, it has been confirmed that there are certain differences in the monitoring of men and women. The weak point of this study could also be the low number of respondents. The limitations of this study can also be seen when creating heat maps. The heat maps were created separately for Group A and Group B with each group containing 19 participants. However, **Pernice and Nielsen (2009)** recommend a minimum of 30 respondents for the creation of heat maps. Therefore, the resulting heat maps shown above may be slightly distorted.

CONCLUSION

The eye-tracking research has shown that color saturation of the fruit juice package has a significant impact on the attention of Y-generation consumers since the *p*-value in three out of four tests of color saturation of the juice package was lower than $\alpha = 0.05$. The lesser attention paid to the juice as the result of a lower saturation effect has been confirmed in up to three out of four juices. This claim is also reinforced by the fact that the vast majority of respondents chose the package with the highest color saturation as the packaging which evoked the highest appetite for orange juice. Adding information to the front side of the juice packages did not significantly reduce the viewing time of the back of the juice packages. Therefore, the importance of reading information on the back sides of juice packages, which is sometimes or occasionally read by up to 85% of the respondents, has been confirmed. The respondents spent most of their time reading the juice composition while viewing the back side. In the case of an image type, its impact on respondents' observation time was not confirmed. It however did have an impact on fancying the orange juice, as up to 90% of respondents evoked the appetite by looking at the images of real oranges. It has also been found out that when deciding on the induction of appetite, the long-term focus was on fruit images on the juice package.

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ANTIOXIDANT ACTIVITIES OF SNAKEHEAD (*CHANNA STRIATA*) FISH SKIN: PEPTIDES HYDROLYSIS USING PROTEASE TP2 ISOLATE FROM SWAMP PLANT SILAGE

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ABSTRACT
The purpose of this research was to study the antioxidants activities of peptides from skin fish of snakehead (*Channa striata*), using hydrolysis of protease TP2 isolate from swamp plant silage. This research 5 treatments hydrolysis time (0, 30, 60, 90, 120 min, respectively), with two replicates, which included several stages of preparation and pre-treatment of the snakehead fish skin production of protease enzymes which were isolated from swamp water, preparation of protein hydrolysates, measurement of hydrolysis degrees, analysis of peptides content and analysis of the antioxidant activity. Results showed that the treatment had given a significant effect on the 5% level of the degree of hydrolysis production (13.98% – 27.08%), with peptides content of 2.73% – 3.78% and antioxidant activity (10.75% – 20.7%). The results of the degree of hydrolysis indicate that the longer the hydrolysis time, the percent degree of hydrolysis will increase. Peptide content and antioxidant activity were increased with increasing hydrolysis time.

Keywords: hydrolysis time; protein hydrolysates; skin; snakehead (*Channa striata*); antioxidant

INTRODUCTION
Snakehead fish in South Sumatra is generally used as basic ingredients of the typical Palembang food industry, namely pempek, kerupuk, and kemplang. The processing process produces waste, one of which is skin waste. The waste is still underutilized due to lack of technological equipment, low commercial value, and a lack of application to the waste **(Blanco et al., 2007)**.

Unused waste contains very important nutritional compounds such as protein content (collagen and keratin) and mineral composition **(Moller et al., 2008)**. This waste has the potential to be used as a protein hydrolysate containing bioactive peptides. Several studies have shown that fish protein hydrolysates have functional properties as antihypertensive, anticancer, antimicrobial, and antioxidant. Bioactive peptides can be obtained by several methods of hydrolysis, namely hydrolysis with digestive enzymes and hydrolysis by proteolytic enzymes produced by microorganisms or plants **(Korhonen, 2009)**.

The bioactive activity of peptides is very diverse and is determined by the sequence of amino acids that make up it. Some bioactive peptides can be precursors of proteins or peptides that will be active when hydrolysed from natural proteins through enzymatic hydrolysis in the digestion, fermentation, and processing processes **(Korhonen, 2009)**. Bioactive peptides have several mechanisms of antioxidants, among others: as radical scavenging (free radical deterrent), a mineral chelating, metal-reducing agents and protectors **(Elias et al., 2008)**. The antioxidant activity of bioactive peptides is strongly influenced by the natural nature and composition of the relevant peptide fragments **(Phelan et al., 2009)**. This is very much determined by the specificity of the protease enzyme used **(Korhonen and Pihlanto, 2006)**. The potential for peptides as antioxidants is not only limited to the prevention of risk factors for degenerative diseases, but also for cosmetic composition and food preservation **(Samaranayaka and Li-Chan, 2011)**.

Proteases are hydrolytic enzymes that can break down peptide bonds between amino acids. Protease enzymes hydrolyse peptide bonds specifically from their original proteins, then produce peptides with sequences and diverse functional properties **(Gonzalez-Rabade et al., 2011)**. One source of proteases are some microorganisms that have been known to produce proteases for commercial applications are *Bacillus, Lactobacillus, Pyrococcus, Termonospora, Rhizopus, Mucor, Endothia* and *Aspergillus* **(Rao et al., 1998)**. In this study using TP2 isolates from swamp plant silage which had a high protease enzyme activity **(Baehaki et al., 2018)**. Protease enzymes are used to hydrolyse proteins in snakehead fish skin and then the hydrolysates produced are tested for antioxidant activity. Antioxidants are known to inhibit the work of free radicals so that the search for antioxidants

from snakehead fish skin is an effort to optimize the use of natural materials in Indonesian waters.

Scientific hypothesis

Protease from swamp plant silage isolates can be used for hydrolysis of snakehead fish skin (*Channa striata*) to produces peptide with antioxidant activity. Degree hydrolysis, peptide content and antioxidant activity increase with increasing hydrolysis time.

MATERIAL AND METHODOLOGY

Materials

The materials used in this study were snakehead fish (*Channa striata*) size ±500 g (weight), TP2 isolate, trichloroacetic acid (Merck, Germany), NaOH (Merck, Germany), DPPH and nutrient Agar (Merck, Germany). The tools used include pH meter, OHAUS analytical balance, incubator, micropipette (Single Channel Capp 10-100 Ul, USA), autoclave (Hirayama, Japan), hotplate (Cimarec, United Kingdom) and spectrophotometer.

Methods

Preparation of Snakehead fish Skin

The preparation of snakehead fish skin is done according to the method of **Liu et al. (2015)**. Preparation is done by separating the skin from other parts such as scales and the rest of the meat. The skin is cut to the size of approximately 1 x 1 cm^2 using scissors. The first stage is the pre-treatment process with NaOH solution which aims to eliminate non-collagen proteins and other impurities such as fat, minerals, pigments, and odours. The fish skin of *Channa striata* is soaked in NaOH solution with a concentration of 0.05 M for 6 h and every 2 h the NaOH solution is replaced with the ratio between the skin and NaOH solution is 1:10 (w/v). The fish skin of *Channa striata* immersed in selected NaOH is washed to near neutral pH.

Refresher Culture

The way the culture of refresher works is as follows: The culture used in this study was in the form of culture stored in the refrigerator. Therefore, the culture must be refreshed first. Approximately 1 ose was scratched after being transferred into a test tube containing 10 mL of sterile NB media, then incubated at 37 ℃ for 24 h. Furthermore, 0.1 mL of the test tube containing the culture was taken and put in another test tube containing 10 mL of sterile LB media to be incubated at 37 ℃ for 24 h. Incubated cultures are ready for use.

Protease Production from TP2 Isolate

The production of protease enzymes uses the method of **Baehaki, Rinto and Budiman (2011)** modified, carried out as follows: TP2 isolates were inoculated on 10 mL of Luria Bertani Broth (LB) media with 1% tripton composition, 1% NaCl, and 0.5% yeast extract. LB media taken 10% of the amount of media then added to the new Luria Bertani Broth (LB) media as a medium for producing proteases. The media is then incubated in the shaker incubator for 45 h, at 37 ℃ at a speed of 120 rpm. The extraction of the protease enzyme was carried out by centrifuging the medium of bacterial growth at a speed of 3000 rpm for 15 min at 4 ℃. The supernatant is an enzyme extract that will be used to hydrolyse protein.

Hydrolysates Production

The preparation of protein hydrolysates was carried out according to the method of **Bhaskar et al. (2008)**. The raw material in the form of fish skin pre-treatment has been mixed with a pH 7 buffer until homogeneous by comparison (1:10). The protease enzyme is added with a concentration of 20% (v/v). The mixture is then hydrolysed at 55 ℃ for 0, 30, 60, 90, and 120 min using a water bath shaker, during the hydrolysis process the sample is stirred regularly. The results of hydrolysis are included in the water bath to inactivate the enzyme at a temperature of 85 ℃ for 20 min. Samples were centrifuged for 20 min at 10 ℃ with a speed of 6000 rpm to separate the dissolved fraction (supernatant) and the non-soluble fraction (pellet). The protein hydrolysate of snakehead fish skin (*Channa striata*) produced was frozen, before being used analysed.

Degree of Hydrolysis.

The degree of hydrolysis is calculated based on the percentage ratio of trichloroacetic acid (TCA) according to the method of **Hoyle and Merritt (1994)**. 20 mL of protein supernatant/hydrolysate added 20% TCA (w/v) as much as 20 mL. Then was centrifuged at a speed of 8,000 rpm at 4 ℃ for 10 min. The resulting supernatant was analysed for protein content. The degree of hydrolysis can be calculated using the following formula:

$$\% \text{ DH} = \frac{\text{100 x dissolved nitrogen in TCA 10\%}}{\text{Total nitrogen in the sample}}$$

Analysis of Peptide Content

Analysis of extract peptide levels was carried out using the formol titration method **(Wikandari and Yuanita, 2016)**, as follows: A total of 5 mL extracts of the sample are put in 100 mL Erlenmeyer. Then extract the sample added 10 mL aqua bides and ±0.5 mL PP indicator. Then the sample was titrated with 0.1 N NaOH until it is pink. Samples added 1 mL of 40% formaldehyde solution and titrated with NaOH.

$$\%N = \frac{a}{B \times 10} \times \text{NaOH} \times \text{Ar N} \times \text{FP}$$

Where:
a = Titration Volume Formol; b = Sample Weight; fp = Dilution Factor

Analysis of Antioxidant Activities with DPPH Method

Testing of antioxidant activity using the DPPH method which refers to **(Shimada et al., 1992)** is as follows: The samples tested for determining the highest antioxidant activity were protein hydrolysate filtrate which was diluted 20 times with ethanol solvent p.a. The sample solution and the comparative antioxidant solution that was made each were taken as much as 1.5 mL and reacted with 1.5 mL of 0.1 mM DPPH solution in a test tube. The mixture is then vortexed and incubated at 37 ℃ for 30 minutes and the absorbance is measured at a wavelength of 571 nm to determine its inhibitory percent. The results of absorbance

measurements and to determine antioxidant activity are expressed in the formula:

$$\% \text{ Inhibition} = \frac{\text{blank absorbance - sample absorbance}}{\text{blank absorbance}} \times 100$$

Statistical analysis

All experiments were carried out in triplicate and the results are reported as means with standard deviations. The experimental data were subjected to analysis of variance (Duncan's test), at the confidence level of 0.05 using the SPSS software (IBM, USA).

RESULTS AND DISCUSSION

Degree of Hydrolysis

The degree of hydrolysis is a parameter that shows the ability of proteases to break down proteins by comparing amino nitrogen with total nitrogen, the degree of hydrolysis can be used as an indicator of the success of the hydrolysis process **(Hasnaliza et al., 2010)**. Hydrolysis conditions are generally influenced by substrate concentration, enzyme concentration, temperature, pH, and time **(Muchtadi et al., 1992)**. Different hydrolysis times will produce different types of free amino acids and peptides which can be seen from the percentage of hydrolysis. The value of the degree of hydrolysis changes during the hydrolysis process. The percentage value of the hydrolysis degree of snakehead fish skin (*Channa striata*) can be seen in Figure 1.

The results of the degree of hydrolysis indicate that the longer the hydrolysis time, the percent degree of hydrolysis will increase. The smallest hydrolysis degree found in the treatment the 0 min hydrolysis time was 13.98% and the highest value of hydrolysis degree in the treatment 90 min hydrolysis time was 27.08%. The degree of hydrolysis of snakehead fish skin increased faster in the first 30 min, however, after hydrolysis time of 30 to 90 min there was an increase but not significant (p <0.05). This might be due to the hydrolysis time that was used not long. In the study of **Gomez-Guillen et al. (2010)**, the hydrolysis level of gelatine hydrolysate of tuna skin and squid skin using alkalase has a maximum hydrolysis degree value of 47.52% incubated for 150 min and 43.46% after incubation for 110 min.

In this study, there was a decrease in the value of hydrolysis degrees in the treatment of 120 min hydrolysis time with a percentage of hydrolysis degree of 24.97%. This is in line with the research of **Khirzin et al. (2015)**, on the hydrolysis of collagen protein peptides from gamma sea cucumber using the pepsin enzyme, decreasing the degree of hydrolysis starting from the treatment when hydrolysis 180 min at 54.54%. Decreasing the degree of hydrolysis was caused by several conditions including a decrease in the concentration of available peptide bonds to be hydrolysed, decreased enzyme activity, and the inhibition of the substrate hydrolysis process by the products produced **(Guerard et al., 2001)**. Literature studies show that there was a relationship between the degree of hydrolysis and its bioactivity, generally its antioxidant activity **(Klompong et al., 2007**) and ACE inhibitors **(Chen et al., 2012)**.

Peptide Content

Peptides are composed of two or more amino acids that form a bond. If the number of amino acids below 50 molecules are called a peptide if more than 50 molecules are called proteins. Bioactive peptides have extensive biological functions and are beneficial for health, which can function as antimicrobial, antihypertensive, antioxidant, anticytotoxic, and mineral transporting activities **(Korhonen and Pihlanto, 2006)**. Fish skin contains collagen which has three polypeptides (α-chains) in the form of triple helix and can be a source of protein needs animal for the body **(Gelse et al., 2003)**. Peptide level analysis was carried out by the formol titration method **(Wikandari and Yuanita, 2016)**.

The purpose of this hydrolysis was to produce peptides with lower molecular weights to produce peptides with higher antioxidant activity. The average value of the peptide content contained in snakehead fish hydrolysate was shown in Figure 2. The results of the research on the determination of peptide content, the average value of protein hydrolysate peptide content of snakehead fish skin produced ranged from 2.73 to 3.78%. The hydrolysis time treatment of 0 min was not significantly different from the hydrolysis time treatment of 30 and 60 min but it was significantly different from the hydrolysis time treatment of 90 and 120 min. This shows that hydrolysis time that was not long (30 and 60 min) produces the same peptide content as without hydrolysis (0 min), but the use of 90 and 120 min of hydrolysis time results in increased peptide content.

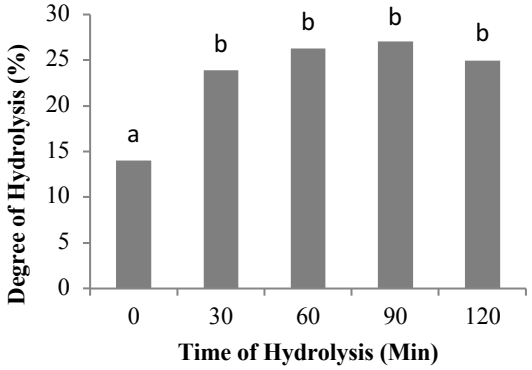

Figure 1 Degree of Hydrolysis of Snakehead Fish (*Channa striata*) Protein.

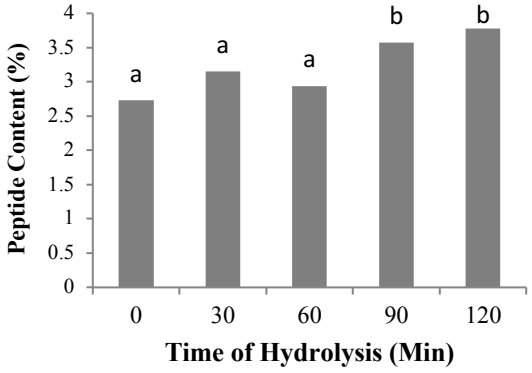

Figure 2 Peptide Content of Hydrolysis Protein from Snakehead Fish (*Channa striata*).

Determination of peptide content was carried out using formol titration, the end point of the titration if the colour changes to pink colour. Peptide bioactivity was influenced by molecular size and amino acid composition **(Gomez-Guillen et al., 2011)**. **Putalan (2018)** states that the hydrolysis time can increase the concentration of peptide content in the selar fish hydrolysate protein. **Nielsen et al. (1997)** also state that peptide content increase as the degree of hydrolysis increases, this was because during the process of hydrolysis the protein was broken down into simpler peptides.

Antioxidant Activity with DPPH Method

In this study, the antioxidant activity of protein hydrolysate of snakehead fish skin was measured using the DPPH method. DPPH which has the molecular formula $C_{18}H_{12}N_5O_6$ and molecular mas was 394.33, DPPH was a stable free radical that can react with other radicals to form more stable compounds **(Molyneux, 2004)**. DPPH can also react with hydrogen atoms to form a stable reduced DPPH (diphenyl picrylhydrazine). A compound can be said to have antioxidant activity if the compound is able to donate its hydrogen atom **(Molyneux, 2004)**. The DPPH method can be used to test solid or liquid samples and is not specific to certain antioxidant components **(Baehaki et al., 2015)**. Percent of DPPH free radical inhibition of snakehead fish skin protein hydrolysate was shown in Figure 3.

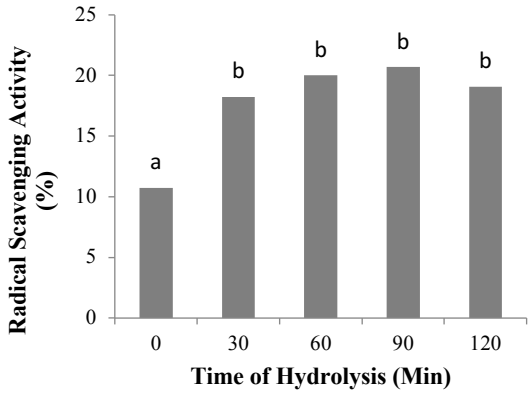

Figure 3 Radical scavenning activity of hydrolyze from Snakehead Fish (*Channa striata*).

The results of the study showed that the highest antioxidant activity produced had a percentage of 20.7% in the treatment of 90 min hydrolysis time. Figure 3 shows the difference between radical scavenging activity (antioxidant activity) without hydrolysis (0 min) and hydrolysis (30 to 120 min). The hydrolysis time used (30 to 120 min) produces radical scavenging activity that was not significantly different (p <0.05), this may be due to the hydrolysis time was used not long and the enzyme was used crude enzyme from TP2 isolate.

Several studies on the antioxidant activity of proteins have been carried out such egg yolk protein **(Park et al. 2001)**, Alaska Pollack skin gelatine hydrolysate **(Kim et al. 2001)**, pork protein **(Carlsen et al. 2003)**, yellowfin fish protein **(Jun et al. 2004)** and collagen from skin fish **(Baehaki et al., 2016)**.

CONCLUSION

Protease from swamp plant silage isolates can be used for hydrolysis of snakehead fish skin (*Channa striata*) to produces peptide with antioxidant activity. The difference in hydrolysis time in the preparation of snakehead fish skin hydrolysates has a significant effect on the degree of hydrolysis, peptide content, and antioxidant activity (p <0.05).

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EFFECT OF INOCULATION ON THE CONTENT OF BIOGENIC ELEMENTS IN THE WHITE LUPINE AND GRASS PEA

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ABSTRACT

The aim of this work was to determine the influence of the inoculant on the content of biogenic elements in ten foreign varieties of white lupine (*Lupinus albus*) and three varieties of grass pea (*Lathyrus sativus* L.) of Slovak origin. Rizobine was used as the inoculum before sowing. Dried and homogenised seed samples were mineralised using concentrated HNO^3 using the MARS X – Press 5 instrument. Analytical determination of macro- and microelements in all samples was performed using ARIAN DUO 240FS/240Z atomic absorption spectrometer. The determined values of biogenic elements content were expressed as $mg.kg^{-1}$ of dry matter. The average content of Cu was lower for both crops in variant A compared to variant B. The addition of the inoculant increased the content of Cu in both crops in lupine by 3.7% and grass pea by 10.94%. The Zn content of variant A in lupine was 19.14% higher than that of the grass pea. Grass pea seeds contained 97.76% less Mn than white lupine seeds in both variants. The Cr content of white lupine was 67.74% higher in variant A than in grass pea. The inoculant also increased the content of Cr in lupine by 25.0%. Lupine contained 30.02% less Fe in variant A and 41.27% less Fe in variant B than the grass pea. The results we have obtained show that Ca, K, and P are the predominant elements in the seeds of grass pea in both variants. By comparing selected types of legumes we found that the grass pea features a higher content of Cu, Fe, K, and P. The analysed seeds of white lupine had a higher content of Zn, Mn, Cr, Ni, Co, Na, Ca, and Mg. In conclusion, inoculation does not significantly affect the content of biogenic elements of selected legume species.

Keywords: lupine; grass pea; inoculation; biogenic elements; analysis

INTRODUCTION

Legumes are the mature, dry seeds of annual and perennial plants of the *Fabaceae* family. Even though legumes have been in our diet for more than a thousand years, studies in recent years have pointed to their unique composition. They are a source of plant proteins, containing a complex of saccharides, soluble and insoluble fibre, essential vitamins, minerals, and polyphenols. The content of these substances advises them among functional foods with a beneficial effect on human health. In combination with cereals, they provide the body with full-value proteins.

Legumes are largely consumed by humans worldwide. Human diets are mostly insufficient in mineral elements like zinc (Zn), iron (Fe), iodine (I) and they may also be deficient in selenium (Se), copper (Cu), calcium (Ca), and magnesium (Mg) mostly in less developed countries **(Welch and Graham, 2002; White and Broadley, 2005)**. Minerals are present in greater quantities in legumes than in cereals. The most represented macroelements are potassium, phosphorus, and calcium. Legumes are rich in iron, zinc, manganese, copper, nickel, cobalt, and molybdenum **(Tichá and Vyzínová, 2006; Velíšek, 1999)**.

Essential elements, including the main elements and a number of trace elements, make up electrolytes, enzymes, vitamins and hormones **(Gruchow, Sobucinski and Barboriak, 1988; Kirbaşlar et al., 2012)**. The importance of mineral composition is due to their nutritional properties and beneficial health effects, as well as their meeting dietary guidelines required for a healthy diet **(Welna, Klimpel and Zyrnicki, 2008; Kirbaşlar et al., 2012)**.

The demand of the ever-growing world population for protein foods is no longer sustainable through animal products alone. To compensate for this deficiency, soya bean has become the prevalent source of plant protein for food and feed.

During recent years, human consumption of lupine seeds has increased worldwide as lupine seeds are a good source of nutrients, not only of proteins but also lipids, dietary fibre, minerals, and vitamins. There are variations in the protein content between species, cultivars, the growing conditions, and soil types **(Martínez-Villaluenga, Friás and Vidal-Valverde, 2006)**. Although lupine belongs to the legumes and is not described as an oilseed crop, it has a considerable amount of oil in its seeds **(Uzun et al., 2007)**. Lupine seeds are a good source of macro- and

microelements **(Ehsan et al., 2015)**. Some *Lupinus* species are able to accumulate Zn, Cd, Mg, Al, Hg **(Esteban et al., 2008)**, Pb, and Cr **(Ximénez-Embún et al., 2001)**; therefore new species of lupine should be examined before its application for food.

Grass pea (*Lathyrus sativus* L.) is an annual plant of the *Fabaceae* family, commonly used for feed or feed components. Grass pea is high in protein (26 – 32%), highly adaptable to extreme conditions, resistant to disease, and has a low moisture requirement for growing. *Lathyrus sativus* L. is cultivated worldwide as a resistant leguminous plant showing tolerance to various biotic and abiotic factors **(Vaz Pato et al., 2006; Nagati et al., 2015)**.

One of the most beneficial characteristics of legumes is their ability to fix atmospheric nitrogen in symbiosis with soil bacteria called rhizobia which form several species **(Peix et al., 2015)**. These bacteria induce nodules in legume roots or stems where the nitrogen fixation takes place after the infection process. This process requires several steps.

After nodule formation, the rhizobial cells are released into the plant cells, and they are transformed into bacteroids which are able to fix atmospheric nitrogen. This process involves plant and bacterial proteins such as leghemoglobins and nitrogenases **(Peix et al., 2010)**. Therefore, the inoculation of a legume with rhizobia induces metabolic changes in the plant. The most studied changes have been the increase in nitrogen and protein contents, which have been exploited in agriculture to improve the crop yield of several legumes **(Morel, Braña and Castro-Sowinski, 2012)**. Also, in the last decades, the increase of other plant components, such as phosphorous, has been studied after the inoculation of phosphate solubilising rhizobia **(Dahale, Prashanthi and Krishnaraj, 2016)** and currently, the increase of potassium by using K-solubilising bacteria is starting to be analysed **(Kumar et al., 2016)**.

It is apparent that the studied legumes are meaningful sources of all the necessary elements in the human diet. The aim of this work was to determine the influence of inoculant on the content of biogenic elements in selected varieties of white lupine (*Lupinus albus*) and grass pea (*Lathyrus sativus* L.).

Scientific hypothesis

We hypothesize that the content of inoculant will have an effect on the content of macro and microelements in white lupine and grass pea.

MATERIAL AND METHODOLOGY

The analysed plant material was sown in field trial plots (GPS coordinates 48.5917973 and 7.827155) at the National Agricultural and Food Center – Research Institute of Plant Production in Piešťany (Figure 1, Figure 2, Figure 3, Figure 4). Piešťany belongs to the corn production area, which is located at an altitude of 162 m.n.m. In this area, the average annual temperature is around 9.2 °C, and long-term precipitation average is 625 mm. The climate is typically lowland, slightly dry, and slightly windy. The average number of sunny days is 265 per year.

The evaluated material consisted of two types of legumes: white lupine (*Lupinus albus*) and grass pea (*Lathyrus sativus* L.). From the selected types of legumes, we analysed 11 genotypes of foreign origin *(Lupinus albus)* and three genotypes of Slovak origin (*Lathyrus sativus* L.) (Table 1), which belong to the gene pool of legumes in the Gene Bank of the Slovak Republic. The weight of each legume average sample was 200 g.

Two variants were sown from each genotype control variant (A) and the variant with inoculant (B). The experimental plot had dimensions of 5.2 x 1.5 m. Rizobin was used to inoculate the legume seeds. It has a high content of live bacteria (5*109). An organic polymer was used as a binder in the formulation. Seed inoculation was performed by simple mixing directly in the seed drill. The advantage of seed inoculation is an increase of 13 – 25% of the crop, but also an increase of some qualitative indicators, such as the content of crude protein and oil. Samples were taken at full maturity, dried, and then cleaned.

Analytical methods used

For the analysis, we used dry seeds of selected types of legumes, 1 g from each genotype. Samples were analysed in two phases. In the first phase, the material was decomposed in a wet way with the addition of 10 cm^3 of oxidised HNO_3.

Mineralisation of the samples was performed by microwave digestion in the Mars X-Press 5 (CEM Corp., USA). In the second phase, after the mineralisation, the residue was filtered and filled with distilled water in a 50 cc volumetric flask to the exact volume. The analytical endpoint of the determination of the heavy metal content in the plant material was atomic absorption spectrometry using the VARIAN DUO 240FS/240Z (Varian. Australia) instrument. All risk metal content values were expressed as $mg.kg^{-1}$ of dry matter in order to assess the safety of the monitored plant food raw materials as objectively as possible. Eachl analysis was done in 4 repetitions.

Statistical analysis

The measured data were statistically evaluated using the statistical package STATGRAPHICS Centurion XVI.II. The t-test was used to compare means, F-test to compare standard deviations, Mann-Whitney (Wilcoxon) W-test to compare medians, and Kolmogorov-Smirnov test to compare the distributions of the two samples.

RESULTS AND DISCUSSION

Legume seeds are known to contain important nutrients like Mn, Cu, P, Zn, Mg, Ca, and K **(Glew et al., 1997; Tharanathan and Mahadevamma, 2003)**. The average values for the content of select macro- and microelements of lupine and pea in the control variant (A) and in the variant with inoculum addition (B) are given in Table 2 and Table 3.

We found that the average content of Zn, Mn, Cr, Ni, and Co was higher in lupine in both variants (Table 2). On the other hand, the content of Cu and Fe were higher in grass peas in both variants (Table 2). Zinc plays a central role in many biochemical and immunological functions **(Fell and**

Lyon, 1994; O´Dell and Sunde, 1997). The Zn content of variant A in lupine was 19.14% higher than that of the grass pea. The addition of the inoculant reduced the Zn content by 16.40% in grass pea. White lupine seeds contained Zn in variant A by 2.87% more than in variant B. Many metabolic processes require magnesium, iron, and potassium.

Similarly, the content of microminerals in the evaluated leguminous plant seeds was dependent on variety too, and the greatest differences were noted for manganese. Noteworthy, the white lupine seeds accumulated significant amounts of manganese **(Page, Weisskopf and Feller, 2006)**. The content of Mn in lupine seeds strongly depends on the field conditions and the species. **Hung et al. (1987)** examined 33 samples of seeds from two species of lupin and found that *L. angustifolius* had a much lower Mn content (61 $\mu g.g^{-1}$) than *Lupinus albus* (1316 $\mu g.g^{-1}$). The average Mn content was higher in white lupine in variant A (571.63 $mg.kg^{-1}$) and also in variant B (588.20 $mg.kg^{-1}$) in comparison to the results of other types of lupine seeds. Generally, the Mn content of *Lupinus albus* L. 2277.16 $mg.kg^{-1}$) was found to be higher than other legume seeds **(Özcan, Dursun and Al Juhaimi, 2013)**. Grass pea seeds contained 97.76% less Mn than white lupine seeds in both variants. The Mn content in variant A was 8.11% lower than in variant B. According to **Trumbo et al. (2001)** the greatest levels of this element were noted in the white lupine seeds (especially in the Butan cultivar – 370 $mg.kg^{-1}$ DM), compared with only 13 – 20 $mg.kg^{-1}$ DM in the lentil, grass pea, common bean, and broad bean seeds ($p < 0.05$) (Table 2). In these investigations, the average content of manganese was 252 $g.kg^{-1}$ DM, while the adequate intake (AI) of this element is 1.8 mg per day^{-1}.

In humans and animals, Cr is an essential nutrient that plays a significant role in the metabolism of glucose, fat, and protein through potentiation of insulin action. There is limited data on which to base tolerable daily intakes for chromium. The Cr content of white lupine was 67.74% higher in the variant A than in grass pea. The addition of the inoculant also decrease the content of Cr in lupine by 25.0%. We also noticed significant differences in Cr content by comparing both variants in lupine. Variant A contained 52.69% more Cr than variant B. It is the highest difference observed in the comparison of variants A and B for all monitored elements. The average content of Cu was higher for both crops in the A variant compared to the B variant (Table 2). The addition of inoculant increased the content of Cu in both crops of lupine by 3.7% and grass pea by 10.94%. Iron is compulsory for haemoglobin and myoglobin synthesis **(Saleh-E-In et al., 2008)**. The Cu content was increased by 27.1% for grasspea in variant A and by 17.47% in variant B. We found that the content of Fe was higher in the seeds of the grass pea in both variants. Lupine contained 30.02% less Fe in variant A and 41.27% less Fe in variant B than the grass pea. The difference in Fe content of variants A and B was 1.64% for white lupine.

Nickel promotes iron absorption and protects against anemia. Nickel is considered synergistic to Fe by promoting its intestinal absorption. The highest content of Ni was found in seeds of white lupine for the A variant (3.99 $mg.kg^{-1}$). The addition of the inoculant reduced the content of Ni in white lupine by 6.02%. Compared to grass pea, the content of Ni was lowered by 56.64% in the variant A and 58.13% in variant B.

Macroelements such as Na, Mg, K, and Ca are essential for a wide variety of metabolic and physiological processes in the human body; therefore they should be included in daily diets to prevent chronic diseases **(Williams, 2006; Alsafwah et al., 2007)**. Sodium and potassium influence acid-base balance and nerve transmittance. Calcium and phosphorus are necessary for the development and appropriate functioning of bones muscles and teeth **(Saleh-E-In et al., 2008)**. Sodium, potassium, and calcium also impact heart functions. Calcium increases heart shrinkage **(Rajurkar and Damame, 1997)**.

The results we have obtained show that K and P are the predominant elements in the seeds of grass pea in both variants. The higher content of P was determined for the variant B in grass pea (Table 3). The content of K in lupin seeds is 11% lower than that in grass pea in both variants with the addition of an inoculant.

Özcan, Dursun and Al Juhaimi (2013) reported a P content in seeds (*Lupinus albus* L.) of 2.719 $mg.kg^{-1}$ and a Ca content from 1.309 $mg.kg^{-1}$ (*C. arietinum* L.) to 2.781 $mg.kg^{-1}$ *(Lupinus albus* L.). According to our results, the P content of lupine is 1999.04 $mg.kg^{-1}$ for variant A, and the addition of the inoculant increased it to 2596.87 $mg.kg^{-1}$.

The results of our analyses show that the Ca content in the white lupine is 2822.60 $mg.kg^{-1}$ for variant A and 3053.51 $mg.kg^{-1}$ for B variant. By comparing selected types of legumes, we found that the grass pea features higher content of K and P. According to **Grela et al. (2017)**, the seeds of the grass pea contain 8.75 – 9.23% K, 1.14 – 1.24% Mg, 0.97 – 1.03% Ca, 4.68 – 5.13% P, 6.98 – 7.95% Cu, 43.52 $mg.kg^{-1}$ Fe, 29.57 – 31.24 $mg.kg^{-1}$ Zn, and 13.29 – 15.31 $mg.kg^{-1}$ Mn. This data suggests that seeds of the grass pea can be a good source of minerals due to the high content of calcium and magnesium.

The analysed white lupin seeds had a higher content of Na, Ca, and Mg (Table 3). The content of Na in variant A was 37.88% higher in the seeds of white lupine compared to the grasspea seed. Variant B (white lupine) contained 68.41% more Na than variant B (grasspea). There was a significant difference between of Ca content of the crops. The lupine contained 90.56% more Ca than the grasspea in variant A. Variant B contained 120.22% more Ca in the lupine compared to the grasspea. By comparing these two crops the Mg values were not significantly changed.

The measured data were statistically evaluated using the statistical package STATGRAPHICS Centurion XVI.II (Table 4 and Table 5).

A statistically significant difference between lupine variant A and lupine variant B was detected for chromium, sodium, and calcium. A statistically significant difference between grass pea variant A and grass pea variant B was not detected for any element.

Table 1 List of genotypes white lupine and grass pea.

Genotypes	Country of origin
White lupine	
Alban	FRA
Astra	CHL
R-933	POL
Satmarean	ROM
Nelly	HUN
POP I	POL
Los Palacios	ESP
Primorskij	RUS
Solnecnyj	USSR
Weibit	DEU
WTD	POL
Grass pea	
Arida	SVK
Krajova z Kralovej	SVK
Cachticky cicer	SVK

Table 2 Average values of the content of selected microelements in lupine (*Lupinus albus*) and grass pea (*Lathyrus sativus* L.) in control variant and variant with addition of inoculant.

Crop	Content of microelements mg.k^{-1}						
White lupine	**Cu**	**Zn**	**Mn**	**Fe**	**Cr**	**Ni**	**Co**
variant (A)	6.48	20.90	571.63	36.61	0.93	3.99	0.30
variant (B)	6.24	20.30	588.20	36.01	0.44	3.75	0.27
Grass pea							
variant (A)	8.23	16.90	12.83	47.60	0.30	1.73	0.27
variant (B)	7.33	16.97	13.87	50.87	0.33	1.57	0.27

Table 3 Average values of the content of selected macroelements in lupine (Lupinus albus) and grass pea (*Lathyrus sativus* L.) in control variant and variant with addition of inoculant.

Crop	Content of macroelements mg.kg^{-1}				
White lupine	**K**	**Na**	**Ca**	**Mg**	**P**
variant (A)	7946.83	163.49	2822.60	1106.46	1999.04
variant (B)	7779.35	223.86	3053.51	1090.66	2127.55
Grass pea					
variant (A)	8998.17	118.57	1481.20	991.30	2596.87
variant (B)	8781.23	132.90	1386.60	986.00	3538.00

Table 4 The amounts of individual elements in lupine plants without the addition of inoculum (lupine A) and with the addition of inoculum (lupine B) with *p*-values derived from t-test, F-test, W-test, and K-S Test.

Element	Lupine A (average ± SD)	Lupine B (average ± SD)	t-test[1] (*p*-value)	F-test[2] (*p*-value)	W-test[3] (*p*-value)	K-S Test[4] (*p*-value)
Cu	6.482 ±0.525	6.236 ±0.631	0.3333	0.5706	0.3560	0.4700
Zn	20.9 ±2.086	20.3 ±1.891	0.4878	0.7615	0.5531	0.8079
Mn	571.627 ±38.968	588.2 ±34.153	0.3014	0.6846	0.2372	0.4699
Fe	36.609 ±2.221	36.009 ±2.007	0.5139	0.7546	0.4693	0.8079
Cr	0.927 ±0.350	0.436 ±0.201	0.0006	0.0966	0.0011	0.0013
Ni	3.991 ±0.727	3.755 ±0.754	0.4630	0.9109	0.4491	0.8079
Co	0.3 ±0.118	0.273 ±0.079	0.5315	0.2134	0.5584	0.2062
K	7946.83 ±579.885	7779.35 ±577.145	0.5050	0.9883	0.5994	0.8079
Na	163.491 ±31.664	223.864 ±41.463	0.0010	0.4084	0.0013	0.0059
Ca	2822.6 ±239.052	3053.51 ±136.617	0.0115	0.0921	0.0215	0.0758
Mg	1106.46 ±67.687	1090.66 ±46.555	0.5308	0.2536	0.5994	0.8079
P	1999.04 ±409.325	2127.55 ±188.249	0.3554	0.0219	0.0878	0.0758

Note: [1]t-test to compare means; [2]F-test to compare standard deviations; [3]Mann-Whitney (Wilcoxon) W-test to compare medians; [4]Kolmogorov-Smirnov test to compare the distributions of the two samples.

Table 5 The amounts of individual elements in grass pea plants without addition of inoculum (grass pea A) and with addition of inoculum (grass pea B) with P-values derived from t-test, F-test, W-test, and K-S Test.

Element	Grass pea A (average ± SD)	Grass pea B (average ± SD)	t-test[1] (*p*-value)	F-test[2] (*p*-value)	W-test[3] (*p*-value)	K-S Test[4] (*p*-value)
Cu	8.233 ±0.737	7.333 ±1.779	0.4635	0.2932	0.6625	0.9963
Zn	16.9 ±0.361	16.967 ±1.617	0.9478	0.0948	1.0000	0.9963
Mn	12.833 ±1.210	13.867 ±2.743	0.5826	0.3257	0.8248	0.9963
Fe	47.6 ±10.892	50.867 ±1.850	0.6355	0.0561	0.6625	0.5320
Cr	0.3 ±0.1	0.333 ±0.231	0.8298	0.3158	1.0000	0.5320
Ni	1.733 ±0.252	1.567 ±0.321	0.5185	0.7600	0.8248	0.9963
Co	0.267 ±0.115	0.267 ±0.153	1.0000	0.7273	0.8222	0.9963
K	8998.17 ±241.531	8781.23 ±327.498	0.4081	0.7046	0.6625	0.9963
Na	118.567 ±13.83	132.9 ±22.324	0.3980	0.5547	0.3827	0.5320
Ca	1481.2 ±222.769	1386.6 ±144.05	0.5702	0.5897	0.6625	0.9963
Mg	991.3 ±52.747	986.0 ±50.854	0.9063	0.9635	1.0000	0.9963
P	2596.87 ±233.377	3538.0 ±665.986	0.0820	0.2187	0.0809	0.0996

Note: [1]t-test to compare means; [2]F-test to compare standard deviations; [3]Mann-Whitney (Wilcoxon) W-test to compare medians; [4]Kolmogorov-Smirnov test to compare the distributions of the two samples.

Figure 1 Field collection of white lupine in our Gene bank.

Figure 2 Genetic resources of Grass pea in field collection in our Gene bank.

Figure 3 Genetic resources of *Lupinus albus* in field collection in our Gene bank.

Figures 4 Grass pea – our regional variety.

Cabrera et al. (2003) and **Özcan, Dursun and Al Juhaimi (2013)** reported that the levels of macroelements in *Fabaceae* were present in the following ranges: 7426 – 16.558 mg.g^{-1} K; 269.75 – 445.81 mg.kg^{-1} Na; 2719 – 5556 mg.kg^{-1} P; 1309 – 2781 mg.kg^{-1} Ca; and 2083 – 2900 mg.kg^{-1} Mg. According to our analyzes, the values of macroelements were lower for Ca, Mg, P in both crops. The pea had a lower Na content and a higher K content than the stated values as stated **Cabrera et al. (2003)** and **Özcan, Dursun and Al Juhaimi (2013)**. White lupine contained more Na. The levels of microelements were as follows: 2.1 – 22.0 mg.kg^{-1} Cu; 22.5 – 152.80 mg.kg^{-1} Fe; 31 – 109 mg.kg^{-1} Zn; and 17.53 – 2277.16 mg.kg^{-1} Mn **(Cabrera et al., 2003; Özcan, Dursun and Al Juhaimi, 2013)** which is consistent with the values obtained in this study.

CONCLUSION

Despite the fact that the inoculation with bacteria of the Rhizobium did not significantly affect the content of biogenic elements in the selected leguminous species, it is a significant part of legume cultivation. Biologically fixed nitrogen positively affects the harvest as well as improving the nutritional status of the next crop. The effectiveness of seed inoculation is dependent on the common interaction of Rhizobii and the soil moisture, temperature, and the nitrate content of the soil.

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COMPARATIVE ANALYSIS OF ANTIOXIDANT ACTIVITY AND PHENOLIC COMPOUNDS IN THE FRUITS OF *ARONIA* SPP.

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ABSTRACT

Chokeberry (*Aronia* Medik.) is a non-traditional fruit plant known as a rich source of biologically active compounds and inhibits the numerous biological activities. We compared the antioxidant activity and phenolic compounds of fruits between widely cultivated *Aronia mitschurinii* (AM-TCH, from Tchekhov district; AM-D, from Dmitrov district; AM-OZ, from Orekhovo-Zuevsky district of Moscow region, Russia) and introduced North American Aronia species (*Aronia arbutifolia* (AA-M), *A. melanocarpa* (AML-M), *A.* × *prunifolia* (AP-M), which have not been planted yet in the arboretum of Main Botanical Garden of the Russian Academy of Sciences (Moscow). Studying samples were collected in their secondary distribution range. Ethanolic extracts were determined for antioxidant capacity (antioxidant activity by DPPH and phosphomolybdenum methods, the total content of polyphenols, flavonoids, phenolic acids) and measured spectrophotometrically. As standards were used Trolox (TE) for antioxidant activities, gallic acid (GAE) for polyphenol content, quercetin (QE) for flavonoid content, caffeic acid (CAE) for phenolic acid content. The antioxidant activity by DPPH method in ethanol extracts of investigated plants was from 6.96 (AM-D) to 8.89 (AM-OZ) mg TE.g^{-1} DW. Reducing the power of investigated extracts exhibited activity from 151.47 (AM-OZ) to 297.8 (AA-M) mg TE.g^{-1} DW. The content of polyphenol compounds determined from 25.98 (AM-TCH) to 54.39 (AA-M) mg GAE.g^{-1} DW, phenolic acids content was from 7.76 (AP-M) to 11.87 (AM-D) mg CAE.g^{-1} DW and the content of flavonoids detected from 8.12 (AM-OZ) to 16.62 (AM-D) mg QE^{-1} DW. Obtained data showed a strong correlation between the content of polyphenol compounds and reducing the power of extracts (r = 0.700), between flavonoids and phenolic acids (r = 0.771) and also between phenolic acids and reducing power (r = 0.753) in Aronia ethanol extracts. Fruits of investigated species of Aronia can be propagated as a source of polyphenol compounds with antioxidant activity and obtained results may use for farther pharmacological study.

Keywords: *Aronia* spp.; antioxidant activity; polyphenols; flavonoids; phenolic acids

INTRODUCTION

The last studies demonstrated that fruit plants are rich in antioxidants and their use can promote human health **(Widén et al., 2012)**. *Aronia mitschurinii* A. K. Skvortsov and Maitul are one of the most famous sources of food polyphenols and antioxidants, its juice has long been used in clinical practice. The natural distribution range of Aronia is located in the eastern part of North America. According to a later nomenclature **(Hardin, 1973)**, the genus Aronia consists of three species: *A. arbutifolia* (L.) Rers., *A. melanocarpa* (Michx.) and their hybrid *A.* × *prunifolia* (Marshall) Rehder. All three species have been introduced in European gardens since the beginning of the XIX century. At the end of the XIX century, *A. melanocarpa* from Germany has been grown in the nursery of I. V. Mitchurin (Tambov province, Russia). There, by the method of "screening in three generations", a man-made *A. mitschurinii* was born **(Vinogradova and Kuklina, 2014)**. It is still unclear whether this taxon arose as a result of macro mutation, or is it a hybrid between *A. melanocarpa* and *Sorbus* spp. Undoubtedly, however, that *A. mitschurinii*, both by morphological and by genetic characteristics, differs so much from the parental *A. melanocarpa*, which is quite correctly described as an individual species **(Skvortsov and Maitulina, 1982; Skvortsov, Maitulina and Gorbunov, 1983)**. Also, based on the morphological properties of *A. mitchurinii* selection work with this species can be successful **(Vinogradova et al., 2017)**. At first, *A. mitschurinii* has been grown as a fruit crop enriched in vitamins and minerals. In the 1960s, the discovery of high content of P-vitamin substances in its berries led to the using of Aronia juice in medical institutions for the treatment of hypertension **(Vinogradova and Kuklina, 2012)**. Now this species began to be tested as a source of antioxidant activity due to the high content of polyphenols **(Mayer-Miebach, Adamiuk and Behsnilian, 2012; Bräunlich, 2013; Taheri et al., 2013; Žlabur Šic et al., 2017)**.

Previous reports about *Aronia melanocarpa* showed that its fruits contain powerful antioxidants (anthocyanidins,

phenolic acids, quercetin glycosides) with different pharmacological activities such as anti-cancer **(Jeong, 2008; Kulling and Rawel, 2008; Olas et al., 2008)**, cardio-protective **(Olas et al., 2010)**, antimutagenic, lipid-lowering, antihypertensive, hepatoprotective, gastroprotective, antimicrobial, radioprotective, immunomodulatory **(Kokotkiewicz, Jaremicz and Luczkiewicz, 2010)**, anti-inflammatory, anti-allergic, antiatherogenic, antidiabetic **(Banjari et al., 2017)**. Fruits of Aronia species contain flavonoids anthocyanins **(Brand et al., 2017)**. In the fresh fruits of *Aronia* spp. was identified carbohydrates (sorbitol, fructose, glucose, sucrose), organic acids (quinic acid, malic acid, ascorbic acid, shikimic acid, citric acid, etc.) **(Sidor and Gramza-Michałowska, 2019)**, polyphenols (neochlorogenic acid, chlorogenic acid, epicatechin, quercetin-3-rutinoside, quercetin, etc.) **(Šnebergrová et al., 2014; Denev et al., 2018)**. Biochemical composition of fresh fruits it's also sugars (10 – 18%), pectin (0.6 – 0.7%), fat (0.14%), ash (0.44%), mineral compounds (high content of K, Zn, Na, Ca, Mg, Fe) **(Kokotkiewicz, Jaremicz and Luczkiewicz, 2010)**. Some results showed that the fruits of *Aronia* spp. rich in antioxidants such as polyphenols, flavonoids, anthocyanins **(Widén et al., 2012; Kapci et al., 2013)**. According to **Kapci et al (2013)**, chokeberry fruits contain four major anthocyanins such as cyanidin-3-galactoside, cyanidin-3-arabinoside, cyanidin-3-glucoside and cyanidin-3-xyloside. Polyphenols in *A. mitschurinii* 'Viking' have been found to consist of cyaniding anthocyanins, proanthocyanidins, flavonols, chlorogenic acid, and neochlorogenic acid **(Oszmianski and Wojdylo, 2005; Slimestad et al., 2005; Koponen et al., 2007)**.

The study of **Suvajdžić et al. (2017)** demonstrated that *Aronia* × *prunifolia* juice and ethanol extracts showed antialgal activity. Moreover, a higher concentration of selected biochemical compounds was identified in the extracts than in juice. The juice from fruits of *A. mitschurinii* has an antimutagenic activity **(Gasiorowski et al., 1997)**, gastroprotective effect **(Matsumoto et al., 2004)**, hepatoprotective activity **(Valcheva-Kuzmanova and Belcheva, 2006)**, cardioprotective and anti-diabetes effect **(Kulling and Rawel, 2008; Denev et al., 2012; Gralec, Wawer and Zawada, 2019)**, anticancer activity **(Sharif et al., 2012)**, anti-inflammatory effect **(Martin et al., 2014)**, antiatherogenic activity **(Daskalova et al., 2015)**. In addition, fruits of *A. melanocarpa* characterized by antioxidant, antimicrobial, and neutrophil-modulating activity **(Denev et al., 2019)**. Production containing chokeberry fruits also characterized. As reported by **Nguyen and Hwang (2016)**, yogurt with *Aronia melanocarpa* juice had high antioxidant activity.

However, there is little information on the polyphenol contents in the other wild species of Aronia. As reported **Denev et al. (2018)**, in *A. melanocarpa* fruits identified organic acids (quinic, malic, ascorbic, shikimic, citric, oxalic, succinic), polyphenols (neochlorogenic acid, chlorogenic acid, epicatechin, quercetin-3-rutiniside, quercetin-3-glucoside, quercetin, anthocyanins, proanthocyanidins), carbohydrates (the main component is sorbitol).

In Europe, these species are not yet cultivated and are only available in collections of botanical gardens **(Vinogradova and Kuklina, 2014)**, although, according to the latest data, they possess economically valuable traits **(Kokotkiewicz, Jaremicz, and Luczkiewicz, 2010)**. In the USA, native *A. melanocarpa*, *A. arbutifolia,* and *Aronia* × *prunifolia* have unique polyphenol profiles warranting further investigation of their comparative nutraceutical and commercial values **(Taheri et al., 2013)**.

Antioxidant properties of *Aronia* spp. can be useful as well in human life as an animal. Investigation of **Bolser et al. (2013)** showed that autumn-migrating birds selected certain of wild-growing plant fruits among which were *Aronia* × *prunifolia* and *A. melanocarpa*. These two species, along with others, had a high concentration of antioxidants as reported in this study.

The purpose of this study was to investigate the antioxidant capacity of Aronia fruits of different origins.

Scientific hypothesis

The first, we assumed (based on the Vavilov's Law about homological rows) that the closely related North American species of the genus Aronia will have the same high levels of antiradical activity as the widely cultivated *A. mitschurinii*. The second, we assumed that native species (from North America) have the same polyphenol contents as plants in the secondary distribution range (from Moscow).

MATERIAL AND METHODOLOGY

Plant materials

In this study, we investigated fruits of plants of *Aronia arbutifolia* (L.) Pers. (AA-M), *A. melanocarpa* (Michx.) (AML-M), *Aronia* × *prunifolia* (Marshall) Rehder (AP-M) in the arboretum of Main Botanical Garden of the Russian Academy of Sciences (Moscow, Russia), which were brought from the USA in the 1980s. Also, we used to study samples of cultivated *A. mitschurinii* A. K. Skvortsov and Maitul from the Tchekhov (AM-TCH) and *A. mitschurinii* from Dmitrov (AM-D) districts of the Moscow region and one sample of naturalized *A. mitschurinii* from the Orekhovo-Zuevsky (AM-OZ) district of the Moscow region.

Chemicals

All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

Sample preparation

0.2 g of dried plant raw material was extracted with 20 mL of 80% ethanol for 2 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for the next measurements: antioxidant activity, polyphenols, and flavonoids.

Antioxidant activity

Radical scavenging assay

The radical scavenging activity of samples was measured using 2.2-diphenyl-1-picrylhydrazyl (DPPH) **(Sanchez-Moreno, Larrauri and Saura-Calixto, 1998)**. The extracts (0.5 mL) were mixed with 3.6 mL of radical solution (0.025 g of DPPH in 100 mL ethanol). The absorbance of the sample extract was determined using the

spectrophotometer Jenway (6405 UV/VIS, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 mg.L^{-1}; R^2 = 0.988) was used as the standard and the results were expressed in mg.g^{-1} Trolox equivalents.

Molybdenum reducing power

Reducing the power of extracts was determined by the phosphomolybdenum method of **Prieto, Pineda and Aguila (1999)** with slight modifications. The mixture of a sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) incubated at 90 °C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/VIS, England). Trolox (10 – 1000 mg.L^{-1}; R^2 = 0.998) was used as the standard and the results were expressed in mg.g^{-1} Trolox equivalents.

Total polyphenol content

Total polyphenol content extracts were measured by the method of **Singleton and Rossi (1965)** using Folin-Chiocalteu reagent. 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Chiocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 min. in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/VIS, England). Gallic acid (25 – 250 mg.L^{-1}; R^2 = 0.996) was used as the standard and the results were expressed in mg.g^{-1} gallic acid equivalents.

Total flavonoid content

Determination of total flavonoids content was conducted using the modified method of **Shafii et al. (2017)**. 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminum chloride, 0.1 mL of 1 M sodium acetate and 4.3 mL of distilled water. After 30 min. in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/VIS, England). Quercetin (0.01 – 0.5 mg.L^{-1}; R^2 = 0.997) was used as the standard and the results were expressed in mg.g^{-1} quercetin equivalents.

The total phenolic acid content

Determination of total phenolic acid content of extracts was carried out using the method of **Farmakopea Polska (1999)**. 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent, 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of distilled water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1 – 200 mg.L^{-1}, R^2 = 0.999) was used as a standard and the results were expressed in mg.g^{-1} caffeic acid equivalents.

Statistical analysis

The statistically treated data are given in tables as the arithmetical mean values and their standard errors. Data were submitted ANOVA and differences between means compared through the Tukey-Kramer test (α = 0.05). Correlation analysis was performed using Pearson's criterion.

RESULTS AND DISCUSSION

Numerous biologically active compounds act as powerful antioxidants, among which different groups of polyphenol compounds that are determined in fruits and berries **(Szopa et al., 2017; Aly, Maraei and El-Leel, 2019; Rodriguez-Werner, Winterhalter and Esatbeyoglu, 2019)**.

Antioxidant activity

As reported **Oszmiański and Wojdylo (2005)**, antioxidant activity level decreased in the following order: juice, fruits, pomace. There are numerous methods to determine antioxidant activity in plant extracts among them radical scavenging assay by DPPH and phosphomolybdenum method (reducing power of extracts) **(Alam, Bristi and Rafiquzzaman, 2013; Moharram and Youssef, 2014; Pisoschi et al., 2016)**. DPPH scavenging activity is the most popular assay to determine antioxidant capacity **(Gralec, Wawer and Zawada, 2019)**.

Radical scavenging assay by DPPH

Our preliminary data show that the DPPH scavenging activity of extracts for all specimens of Aronia was 83.25 – 93.30% (methanol extracts), 78.07 – 93.23% (ethanol extracts) and 59.87 – 88.36% (aqueous extracts). Alcoholic and aquatic extracts of fruits have almost equal antioxidant activity. The lowest antioxidant activity in alcohol extracts was shown by cultivated *A. mitschurinii*, and the highest one by invasive plants of *A. mitschurinii*. Conversely, aqueous extracts have the lowest antioxidant activity in invasive plants of *A. mitschurinii* and the highest one in cultivated samples **(Vinogradova et al., 2018)**. Also, in another study, the Aronia extract was tested for various antioxidative potentials and inhibitory effects. Thus, high polyphenol and flavonoid content suggests that Aronia extracts may be useful for the prevention or treatment of allergic disease **(Jeong, 2008)**. In study **Jakobek et al. (2012)** antiradical activity of wild chokeberries was higher than cultivars (besides one cultivar).

In our study radical scavenging activity of the ethanol extracts of investigated Aronia species was screened against DPPH radical which is the most used to determine the antiradical activity of several natural compounds and was from 6.96 (AM-D) to 8.89 (AM-OZ) mg TE.g^{-1} (Figure 1). **Strugała and Gabrielska (2014)** determined that significantly higher antioxidant activity was found at the start of storage for Japanese quince and chokeberry extracts than the same of hawthorn and quince. **Tolić et al. (2015)** found that antioxidant activity by DPPH method in dried fruits of *A. melanocarpa* was 183.52 – 191.31 mg TE.L^{-1}. Antioxidant activity by DPPH-method, according to **Wangensteen et al. (2014)**, in methanol extracts of *A. melanocarpa* cultivars and *Aronia* × *prunifolia* was higher than in ethanol extracts. **Yang, Kim and Shin (2019)** measured antioxidant activity by DPPH method of fruit extracts with ascorbic acid (AA) as equivalent and obtained values 4144.44 – 4565.28 mg AA.100 g^{-1} FW.

Molybdenum reducing the power of extracts

We determined the reducing power of Aronia extracts by phosphomolybdenum method that is also widely used to determine the antioxidant capacity **(Alam, Bristi and Rafiquzzaman, 2013)**. We found that reducing the power of ethanol extracts of Aronia samples in our study was from 151.47 (AM-OZ) to 297.8 (AA-M) mg TE.g^{-1} (Figure 2).

It is very difficult to compare data with other reviews because were used different methods of measure and various units. So, **Rugină et al. (2012)** determined reducing the power of extracts by the FRAP method and found a correlation between this parameter and total flavonoid content. The same method of reducing power determination used by **Tolić et al. (2015)** and a strong correlation found between reducing power and flavonoid content. **Oszmiański and Lachowicz (2016)** also used the FRAP method for the determination of reducing the power of extracts and determined a strong correlation between phenolic acids and FRAP and total phenolics and this parameter. Our preliminary results about another species *Asimina triloba* (L.) Dunal. showed molybdenum reducing the power of extracts from 97.25 to 275.41 mg TE.g^{-1} DW **(Brindza et al., 2019)**.

Total polyphenol content

The polyphenols contained in fruits are of great interest for their antioxidant and anti-inflammatory properties **(Chrubasik, Li and Chrubasik, 2010; Montrose et al., 2011)**. Previous data showed that Aronia pomace had higher content of polyphenols than juice and fruits. Polymeric proanthocyanins are the major class of phenolic compounds of fruits of these species **(Oszmiańsky and Wojdylo, 2005)**. As emphasized by **Jurikova et al. (2017)**, the content of total polyphenol compounds and their selected groups of *A. melanocarpa* depends on some factors, among which is the temperature of storage. So, after six months of storage at 3 ℃ content of polyphenols decreased by 30%.

The antioxidant capacity of plant extracts has been attributed to their phenolic contents that were determined by Folin-Ciocalteu reagent. The amount of total polyphenol content of investigated extracts was from 25.98 (AM-TCH) to 54.39 (AA-M) mg GAE.g^{-1} (Table 1).

According to **Denev et al. (2018)**, total polyphenol content was found from 1022.4 to 1795.5 μmol TE.g^{-1} FW. HPLC analysis identified total polyphenol compounds in crude extracts of *A. melanocarpa* fruits 53.5 mg.g^{-1} **(Denev et al., 2019)**. As reported **Gao et al. (2018)**, *A. melanocarpa* polyphenols showed no cytotoxic effect. Polyphenol compounds from *A. melanocarpa* demonstrated immunomodulatory and anti-inflammatory functions **(Ho et al., 2014)**. The main group of *A. melanocarpa* polyphenols is procyanidins **(Gralec, Wawer and Zawada, 2019)**.

According to **Brand et al. (2017)**, the highest concentration of total phenolic compounds identified in fruits of *Aronia* × *prunifolia*, whereas, for *A. arbutifolia* and *A. melanocarpa* this parameter was less. Comparing obtained data and previous results of yogurt with chokeberry juice (*Aronia melanocarpa*), it's should be noted that total polyphenol content of 1%, 2%, and 3% yogurts was 28.70, 41.30 and 54.05 mg GAE.g^{-1} DW respectively **(Nguyen and Hwang, 2016)**. Also, dried fruits of *A. melanocarpa* showed total phenolics content in the range from 2,000 to 8,000 mg per 100 g **(Kokotkiewicz, Jaremicz and Luczkiewicz, 2010)**. In our case, the content of polyphenol content of investigated fruits of *A. melanocarpa* was 40.06 mg GAE.g^{-1} DW. In the study of **Jakobek et al. (2007)** total polyphenol content of *A. melanocarpa* fruits was 10637.20 mg GAE.kg^{-1} and total flavanol content by HPLC method was 76.43 mg.kg^{-1}. Different cultivars of *A. melanocarpa* had polyphenol content from 11721.7 to 14350.3 mg GAE.kg^{-1} FW **(Jakobek et al., 2012)**. As identified by **Jurikova et al. (2017)**, the total polyphenol content of *A. melanocarpa* can vary from 690 to 2560 mg GAE.100g^{-1} FW. In the study of **Rugină et al. (2012)** total phenolic values were 1586.5 to 2059.5 mg GAE.100 g^{-1} FW.

In another study, represented that the total phenolic content of dried fruits of *A. melanocarpa* was 1954 – 2466 mg GAE.100 g^{-1} **(Tolić et al., 2015)**. Investigation of **Veljković et al. (2014)** demonstrated that *A. melanocarpa* tea diffusions, as well as dry or fresh fruits, had a high antioxidant activity due to the content of polyphenol content such as flavonoids and phenolic acids. **Wangensteen et al. (2014)** determined that the polyphenol content of *A. melanocarpa* cultivars was 98 – 148 mg GAE.g of extract and 1079 – 1921 mg GAE.g FW, and for *Aronia* × *prunifolia* this parameter was 175 mg GAE.g and 2996 mg GAE.g FW. **Yang, Kim and Shin (2019)** found that the phenolic content of fruits of Aronia cultivars was 3955.28 – 4393.50 mg GAE.100 g^{-1} FW.

The total phenolic acid content

Phenolic acids are a group of polyphenol compounds that are generally described as phenolic compounds that have one carboxylic acid group and possess higher antioxidant activity than vitamins and present in food mostly in bound form. These compounds exhibited the following activities: antioxidant, antimicrobial, anticancer, anti-inflammatory, anti-diabetic, neuroprotective **(Kumar and Goel, 2019)**. They determined in various berries and fruits such as saskatoon chokeberry, blueberry, dark plum, elderberry, cherry **(Mattila, Hellström and Törrönen, 2006; Jakobek and Seruga, 2012; Lachowicz et al., 2017; Horčinová Sedlačková et al., 2018)**.

The phenolic acid content of Aronia samples in our study determined from 7.76 (AP-M) to 11.87 (AM-D) mg CAE.g^{-1} (Table 2). *A. melanocarpa* is known as a rich source of anthocyanins and phenolic acids **(Oszmiańsky and Lachowicz, 2016)**. The phenolic acid concentration was higher in juice than in pomace of this species whereas total phenolic content is higher in pomace than in fruits and juice **(Oszmiańsky and Wojdylo, 2005)**. In selected cultivars of *A. melanocarpa* identifies high content of chlorogenic, caffeic, ferulic acids. During the process of pasteurization, the most unstable was hydroxycinnamic acid (**Jurikova et al., 2017**). According to **Jakobek and Seruga (2012)**, the phenolic acid content in chokeberry fruits was 266.9 mg.kg^{-1}.

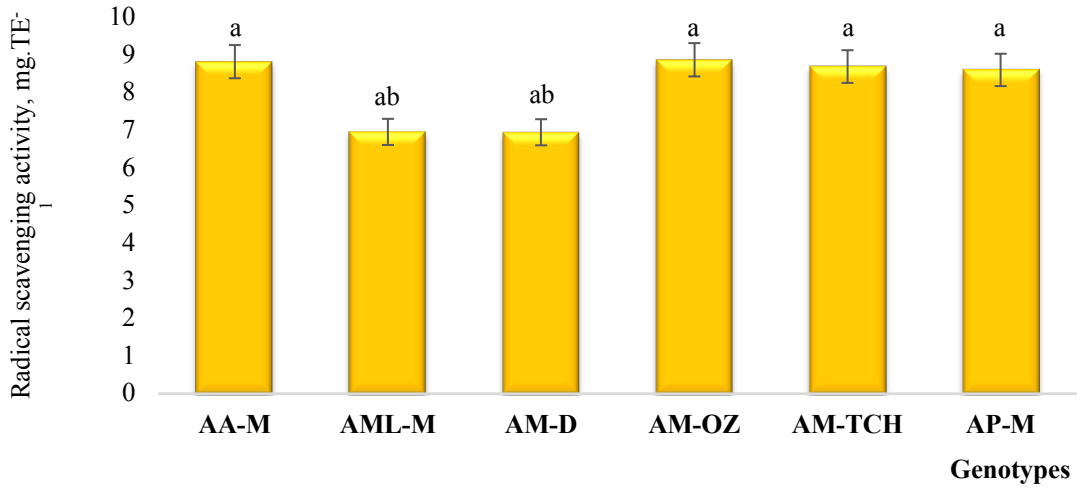

Figure 1 Antioxidant activity by DPPH-method of ethanol extracts of *Aronia* spp. Note: means in columns followed by different letters are different at $p = 0.05$; each value represents the mean of three independent experiments (±SD).

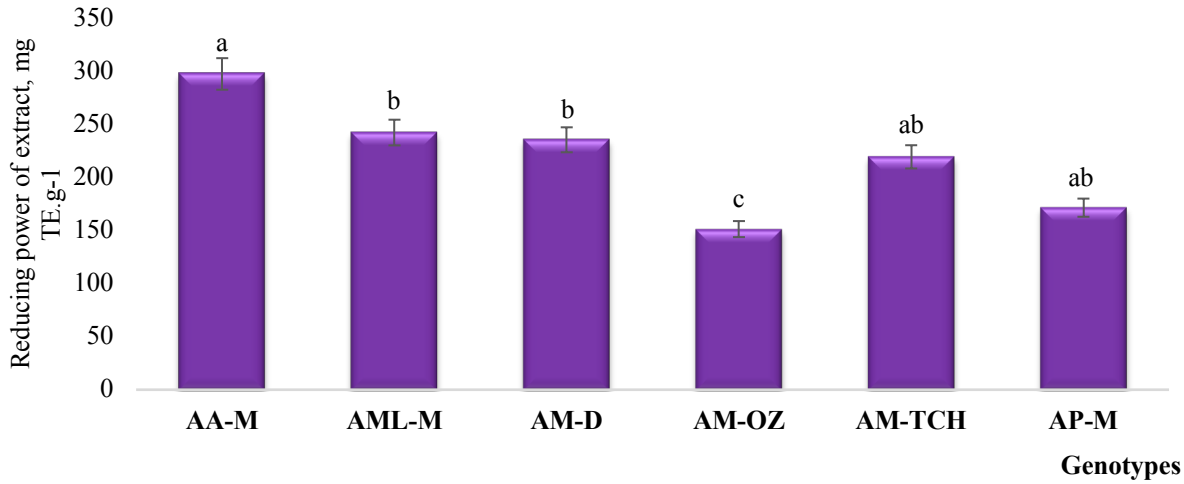

Figure 2 Reducing power of ethanol extracts by phosphomolybdenum method of *Aronia* spp. Note: means in columns followed by different letters are different at $p = 0.05$; each value represents the mean of three independent experiments (±SD).

Table 1 Total polyphenol, flavonoid and phenolic acid content in the fruits of Aronia specimens.

Plant sample	Total polyphenol content, mg GAE.g^{-1}	Total phenolic acids, mg CAE.g^{-1}	Total flavonoid content, mg QE.g^{-1}
A. arbutifolia **(AA-M)**	54.39 ±1.05a	11.06 ±0.79ab	10.88 ±0.41ab
A. melanocarpa **(AML-M)**	40.06 ±1.61b	11.54 ±0.09ab	13.16 ±0.15b
A. mitschurinii **(AM-TCH)**	25.98 ±1.28c	8.79 ±0.42b	11.05 ±0.21ab
A. mitschurinii **(AM-D)**	38.91 ±4.56ab	11.87 ±0.23a	16.62 ±0.50a
A. mitschurinii **(AM-OZ)**	28.80 ±2.75c	8.69 ±0.47b	8.12 ±0.17b
A.* × *prunifolia **(AP-M)**	40.39 ±1.78b	7.76 ±0.31c	10.02 ±0.56c

Note: Means in columns followed by different letters are different at $p = 0.05$; each value represents the mean of three independent experiments (±SD); GAE – gallic acid equivalents; CAE – caffeic acid equivalents; QE – quercetin equivalents.

Table 2 Coefficient of correlation between investigated parameters of *Aronia* spp. extracts.

Characters	Polyphenols	Phenolic acids	Flavonoids	Antioxidant activity (DPPH)	Molybdenum reducing power
Phenolic acids	0.495*	1			
Flavonoids	0.210*	0.771*	1		
Antioxidant activity (DPPH)	-0.098	-0.738	-0.870	1	
Molybdenum reducing power	0.700	0.753*	0.480*	-0.266	1

Note: Significant according to the t-test (p <0.05).

Total flavonoid content

The main groups of flavonoids of *A. melanocarpa* were anthocyanins, proanthocyanins, flavonols and flavanols **(Jurikova et al., 2017)**. Total flavonoid content was determined in the range from 8.12 (AM-OZ) to 16.62 (AM-D) mg QE.g^{-1}. Investigation of **Kapci et al. (2013)** reported that among different products from chokeberry dried fruits had the highest total flavonoids content and antioxidant activity values. But total phenolics and anthocyanins were found in chokeberry pomace. According to **Gralec, Wawer and Zawada (2019)**, the content of flavonoids of *A. melanocarpa* was from 7 to 11 g.100^{-1} DW. The study of **Rugină et al. (2012)** showed that the content of flavonoids ranged from 47.67 to 64.04 mg QE.100 g^{-1} FW. As reported by **Slimestad et al. (2005)**, the black chokeberries contained up to 71 mg flavonols per 100 g FW. **Tolić et al. (2015)** determined total flavonoids content in dried fruits of *A. melanocarpa* 867 – 1394 mg GAE.L^{-1}. According to **Yang, Kim and Shin (2019)**, the flavonoid content of three cultivars of Aronia was 3175.52 to 3577.7 mg CE.100 g^{-1} FW (catechin equivalent).

The antioxidant activity of investigated raw can be caused by the presence of different groups of compounds such as phenolics. In this case, we determined correlations between of investigated parameters. Correlation analysis found a strong correlation between the content of polyphenol compounds and reducing the power of extracts (r = 0.700), between flavonoids and phenolic acids (r = 0.771) and also between phenolic acids and reducing power (r = 0.753) of Aronia ethanol extracts (Table 2). The moderate correlation found between the accumulation of flavonoids and reducing the power of extracts (r = 0.480) and between polyphenol content and phenolic acids (r = 0.495). Weak relation was determined between the accumulation of polyphenol content and flavonoids of investigated extracts. Between rest, parameters were identified as a negative correlation.

As reported by **Gralec, Wawer and Zawada (2019)**, antioxidant activity by the DPPH test of *Aronia melanocarpa* positively correlated with the content of polyphenol compounds of both ripe and unripe fruits. **Yang, Kim and Shin (2019)** indicated a very strong correlation between flavonoid and phenolic content in fruit extracts (r = 0.991), between flavonoids and antioxidant activity by DPPH method (r = 0.997), between total phenolics and chlorogenic acid (r = 0.940). **Tolić et al. (2015)** found that the correlation between reducing power and total phenolic content of chokeberry juice was higher than between total antioxidant capacity and phenolic content.

CONCLUSION

Aronia species are non-traditional fruit plants, which are a rich source of polyphenol compounds with high antioxidant activity. In this study, cultivated plant species had the least values of polyphenol compounds as opposed to naturalized plants. However, the content of flavonoids and phenolic acids varied for all species. It was found that maximal values of polyphenol compounds were determined for *A. arbutifolia*, and total flavonoid and phenolic content for *A. mitschurinii* (from Dmitrov region). Thus, naturalizing plants of Aronia species (*Aronia arbutifolia*, *A. melanocarpa*, *Aronia* × *prunifolia*) can be an alternative source of antioxidants to widely cultivated species (*A. mitschurinii*). Thus, in the secondary distribution range, introduced species retain their biochemical characteristics.

Apparently, plants accumulate fewer biologically active substances in a comfortable culture environment than during forced adaptation to unfavorable ecological conditions. The determination of the polyphenol contents in the closely related species of the genus Aronia is necessary for the further use of the nutrient properties of this small fruit crop. Wild, still exploitable Aronia species could be selectively used to improve the nutritional and functional properties of cultivated *A. mitschurinii* fruits. In addition, interest in functional foods and food additives, which may support the protective mechanisms against diseases caused by oxidative stress, is increased currently.

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RATIONAL AND IRRATIONAL BEHAVIOR OF SLOVAK CONSUMERS IN THE PRIVATE LABEL MARKET

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ABSTRACT
Rational and irrational consumer behavior has already been the subject of several studies. Although their attention was mostly focused on traditional brands, these studies and their conclusions may also serve as a model for private label research, which is now increasingly coming to the forefront. Private labels are gradually becoming an adequate purchase alternative to traditional brands. The aim of the paper was to find out the influence of packaging and chosen marketing communication tools, on consumer purchasing decisions in the dairy segment. An anonymous questionnaire survey was chosen as the main research method, where participated 1,116 respondents from all over Slovakia; which was subsequently supplemented by the blind test involving 20 respondents under the age of up to 25 years. Seven chocolate-flavored yogurt samples were examined in the blind test; while the samples were such traditional brands by traditional producers as well as private labels. Interestingly, identical products were investigated – products from the traditional producer, both under its brand and under the retailer's private label. The results of our research proved some interesting findings – more than 28% of respondents buy private label products regularly and more than 53% buy them sporadically; whereas respondents do not buy private labels, mainly because of their lack of interest to try something new, assuming poor product quality and inadequate price; they buy them mainly because of lower prices compared to traditional product brands, comparable quality to traditional brands and good experience; respondents perceive private labels as good products of adequate quality and private labels evoke that products are adequate quality at a reasonable price. Although most respondents have no opinion on the packaging of private label products, more than 31% of them consider their packaging as unattractive and almost 36% think that their packaging does not affect them, but the results of the blind test partially refused this opinion.

Keywords: private label; traditional brand; marketing communication; packaging; consumer; blind test

INTRODUCTION
One of the modern and probably the most successful strategies of foreign as well as domestic retail chains and companies is to reach as many customers as possible, not only those customers for whom the price is decisive but also those who prefer high-quality products when purchasing. All these requirements are to be met by private labeled products, whose share of household spending in Europe, especially in Slovakia, is constantly increasing (Figure 1).

Private labels, also referred to as store brands, retail chain brands, custom brands, etc. are a traditional retailer branding strategy (whether in its name or wholly owned by the retailer) were firstly introduced in the US at the end of the 19th century **(Nagyová and Košičiarová, 2014)**.

Even though it is not an absolute "novelty", attention is drawn especially at present, when so-called global phenomenon **(Herstein and Gamliel, 2004; Smith and Bashaw, 2009)** and when traders are increasingly aware of their hidden advantage, which is the possibility of strengthening their competitive advantage, resp. higher profits at lower costs. Many studies point to the fact that private labels are more profitable than traditional brands themselves **(Heijn, 2012; Dulíková, 2012; De Wulf et al., 2005)**.

Private labels bring benefits to the retailer as well as to the manufacturer and the customer. The main advantages on the part of the retailer are in particular – strengthening its competitive advantage and achieving higher profits at lower costs. On the customer's side, the biggest advantage is that private labels are an alternative to traditional brand products at comparable, if not higher quality, but lower prices. As far as the manufacturer is concerned, the biggest advantage is the volume of sales and the resulting profits. However, it is necessary to add the fact that private labels also bring many disadvantages such as the possibility of damaging the company´s reputation by a lower-quality product, resp. of knowledge of the original producer, which in many cases

discourages the purchase itself **(Košičiarová, Holotová and Nagyová 2014)**.

Even though the United States (USA) is considered to be the "birthplace" of private labels, in the case of Europe and especially the Slovak Republic it is possible to view a later, but more extensive and faster development and establishment of private labels on the consumer market as in the USA, the share of private label purchases in household expenditures is 18% on average **(Košičiarová and Nagyová, 2015)**; in the case of Europe and the Slovak Republic, we notice three times higher share on average (Figure 1). The speed and rate of private label expansion in Europe, compared to the US, was not only supported by customers' interest, but also by the invention of retail chains that discover much more in private labels than just another commercial item that hides margin **(Augustín, 2005; De Wulf et al., 2005)** - private labels are becoming a source of competitive advantage and in particular, the means by which they can build resp. improve the company´s image. For the customer, on the other hand, private labels are an alternative to traditional brands, but this is no longer exclusively due to the price sensitivity of consumers, but to increasing the quality of private labels products and realizing of their hidden benefits.

This paper deals with the issue of the private labels in terms of their attractiveness for the customers themselves, as it was already indicated above, the private labels are the alternative to traditional brands. The paper intends to find out, to what extent the packaging affects the purchasing decision of the consumers and which communication tools could be used to reach customers´attention to purchase the private label products.

Scientific hypothesis

For a deeper analysis of the research objectives, the following hypotheses were formulated:

Hypothesis 1: We assume there is a correlation between the quality rating of private label products and the gender of respondents.

Hypothesis 2: We assume there is a correlation between the purchase of private label products and the gender of respondents.

Hypothesis 3: We assume there is a correlation between the purchase of private label products and the economic activity of respondents.

Hypothesis 4: We assume there is a correlation between the perception of private label packaging and the gender of respondents.

Hypothesis 5: We assume there is a correlation between the perception of facts that influence respondents to buy private label products and the gender of respondents.

Hypothesis 6: We assume there is a correlation between the perception of facts that influence respondents to buy private label products and the age of respondents.

Hypothesis 7: We assume there is a correlation between the decisive factor when buying private label products and the gender of respondents.

Hypothesis 8: We assume there is a correlation between the decisive factor when buying private label products and the age of respondents.

Hypothesis 9: We assume there is a correlation between the facts that discourage respondents from buying private labels and the gender of respondents.

Hypothesis 10: We assume there is a correlation between the facts that discourage respondents from buying private labels and the age of respondents.

MATERIAL AND METHODOLOGY

The paper aimed to find out how the packaging and selected marketing communication tools affect consumer purchasing decisions in the dairy segment. An anonymous questionnaire survey was chosen as the main research method, with a total of 1,116 respondents from all over Slovakia (Table 1). This sample of respondents can be considered as representative on the 95% confidence level and 3% error margin as $n \geq 1{,}067.12$. The questionnaire survey consisted of 10 questions formulated as closed ones with the possibility of one, resp. multiple responses; and 7 classification questions.

A blind test method was chosen as a complementary method of research, in which participated 20 respondents aged up to 25 years – respondents had to evaluate on a scale of 1 to 5, with 1 being the best and 5 the worst, the color, flavor, fragrance, consistency, and the chocolate ratio in the first round of testing; and color, flavor, fragrance, consistency, chocolate ratio, the attractiveness of the packaging and the grammage in the second round of testing. Total of seven chocolate-flavored yogurt samples were examined in two rounds. Thus, the samples represented traditional brands as well as private labels. Interestingly, identical products were investigated, i.e. products of a traditional producer, both under its brand and under the retailer's private label. Table 2 provides a more detailed overview of the examined yogurt samples.

Statistical analysis

The results of both the questionnaire survey and the blind test were verified by statistical verification of dependencies and the following methods: Pearsons´ Chi-Square Test, Mantel-Haenszel Chi-Square Test, Phi Coefficient, Cramer´s V Coefficient and correspondence analysis by statistical programs XL Stat, SAS Enterprise Guide and SAS 9.4.

In hypothesis testing, if the *p*-value is lower than a significant level, in our case 0.05, the null hypothesis is rejected and the alternative hypothesis is confirmed.

RESULTS AND DISCUSSION

Although rational resp. irrational behavior of consumers has already been studied by various researchers, resp. experts in the field, such as **Galbraith (1938)**, **Hanf and von Wersebe (1994)**, **Dean and Croft (2009)**, **Marsden and Littler (1998)**, **Hantula (2012)**, **Rybanská, Nagyová, and Košičiarová (2015)**, **Guziy, Šedík and Horská (2017)**, **Šedík et al. (2019)**, **Rybanská, Košičiarová and Nagyová (2019),** etc., resp. that the effect of packaging on shopping behavior or the attractiveness and effectiveness of the packaging has been examined by other researchers, e.g. **Horská et al. (2018)**, **Nagyová et al. (2018)**, **Bogdanovičová and Jarošová (2015)**, **Pavelková and Flimelová (2012)**,

Čanigová et al. (2018); it can still be stated that the present contribution represents an innovative approach to this issue since the effect of the packaging on the consumer's purchasing decision in the case of private labels has not been yet examined. In the field of private labels, attention has been, so far, focused primarily on whether consumers buy the products, what product categories do they buy and what benefits these products bring to them or whether consumers prefer traditional brand or private label products **(Hoch and Banerji, 1993; Narasimhan and Wilcox, 1998; Cotterill, Putsis and Dhar, 2000; Steenkamp, Van Heerde and Geyskens, 2010; Košičiarová, Holotová and Nagyová, 2014; Košičiarová and Nagyová, 2015; Košičiarová et al, 2018)**.

As stated in Table 1, the majority of respondents were represented by female gender (63.3% of respondents), aged up to 25 years (49.2% of respondents), with secondary education with A level (39.2% of respondents), employed (48% of respondents), with a net money income of households between 501 and 800 € per month (25%) and city as the residence of respondents (63.9%).

As the aim of the paper was to find out how the packaging and selected marketing communication tools affect the consumer purchasing decisions in the dairy segment, within the questionnaire survey were formulated questions related to the issue of private label products, followed by the blind test carried out in two rounds, to determine whether the packaging influence the purchasing decision of consumers.

In terms of purchasing the private label products, the current situation in Slovakia is favorable, as of total 1,116 respondents, up to 28.7% buy the products on a regular basis and 53.1% of respondents buy them sporadically. For specific reasons for purchasing or non-purchasing of the given products can be said that while our respondents do not buy private labels mainly because of *lack of interest to try something new* (32.3% of respondents), *assumed low product quality* (27.1% of respondents) and *unsatisfactory price* (13.5% of respondents); they buy them mainly because of *lower prices compared to traditional product brands* (31% of respondents), *comparable quality to traditional brands* (24.3% of respondents) and *good experience* (24.3% of respondents). Based on the above, it can be said that rational reasons prevail over irrational ones, and therefore in this case respondents behave rationally.

In terms of the frequency of private label products purchases, our respondents buy the products mainly once a month (27.7% of respondents) and multiple times a week (24.9% of respondents); they buy private brands the most often in the categories of *milk and dairy products, meat and fish* purchased every week, *savory snacks and mineral waters, lemonades, and juices*, which they buy on a monthly or weekly basis. For this reason, dairy products, namely yogurts, were further tested in the blind test.

These results largely correspond to those of research agencies TNS Slovakia **(TNS, 2015)** and GFK Slovakia **(TASR, 2010)**, resp. our previous study **(Nagyová and Košičiarová, 2014; Košičiarová et al., 2017**), which says that Slovaks buy private label products mainly several times a week, resp. once a week; they buy them mainly because of their cost-effectiveness, quality and confidence; and that every Slovak household has in its regular purchases a "favorite brand", for some categories of goods there is a stronger brand preference, when the brand is put before the price, mainly in dairy products that are purchased every third day on average; coffee and coffee specialties followed by margarine and butter, body and dental hygiene products, skin care, shampoos and other hair care products. The survey further shows that, while in a case of long-life milk the “less prestigious“, private label products make up almost 80% of total consumption, within the acidophilic milk, fresh milk and fresh cheeses dominate the traditional brand products **(TASR, 2010)**.

In terms of the perception of quality and evoked impression in consumer perception and consciousness, we can further say that our respondents perceive private labels as products of good resp. of adequate quality (48.7% of respondents), resp. private labels evoke that they are products of adequate quality at a reasonable price (59.8% of respondents).

As has been indicated before, the aim of the paper was also to find out how packaging affects consumers' purchasing decisions. For this reason, the questionnaire survey also formulated questions about how respondents perceive the packaging of products labeled with a private label and whether they think, that packaging influences them in their purchasing decision. The evaluation of the questions showed that although most respondents have no opinion on the packaging of private label products (up to 45.3% of respondents), up to 31.3% think that this packaging is unattractive and up to 35.8% believe that the packaging does not affect them. Following the findings, a correspondence analysis was created in SAS 9.4 to determine if the packaging affected our respondents' purchasing decisions. As can be seen in Figure 2, those respondents who reported that private label products buy regularly think that private labels have attractive packaging and that packaging does not affect them in their purchasing decisions; respondents who buy private labels sporadically think that their packaging is unattractive, resp. that the packaging affects them; and those respondents who do not buy private label products, do not have any opinion.

These findings are followed by a blind test conducted on a sample of 20 respondents from the category of young people (up to 25 years), as they represent potential regular customers of the products. Yogurts have become the subject of the blind test, as several studies proved that private label products are purchased primarily in the categories of milk and dairy products **(Košičiarová et al., 2018)**. The blind test took place in two separate rounds. While in the first round, respondents tasted yogurts without knowing what specific yogurt it was, in the second round they were already tasting yogurts by seeing their particular packaging.

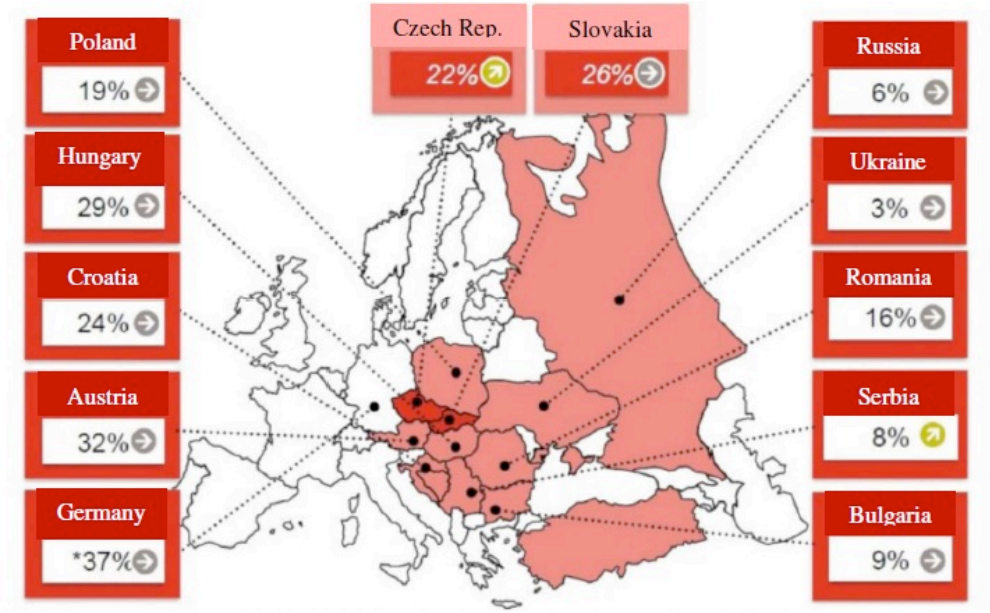

Figure 1 Percentage of Purchases of Private Label Products in the Household Expenditures in 2018 (in %) **(Horáček, 2019)**.

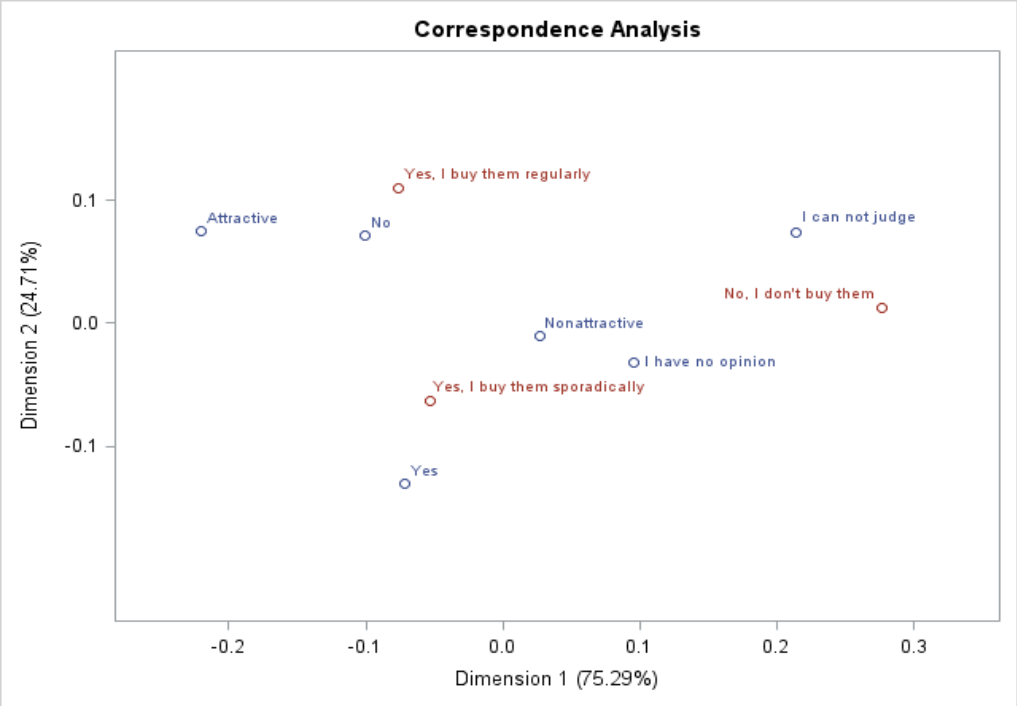

Figure 2 Correspondence Analysis. Note: Source: Results of own research.

Table 1 Characteristics of Respondents.

Gender of Respondents	**Number**
Female	706
Male	410
Age Structure of Respondents	**Number**
Up to 25 years	549
26 – 35 years	231
36 – 45 years	139
46 – 55 years	125
56 years and more	72
Educational Structure of Respondents	**Number**
Primary education	34
Secondary education without A level	80
Secondary education with A level	438
Univesrity education – B achelordegree	334
Univesrity education – Masters degree	226
Other	4
Economic Activity of Respondents	**Number**
Student	392
Employed	536
Unemployed	31
Self-employed	61
Maternity leave	41
Retired	55
Net Money Income of Households per Month	**Number**
Up to 500 EUR	190
501 – 800 EUR	279
801 – 1.100 EUR	253
1.101 – 1.500 EUR	273
More than 1.501 EUR	121
Place of Residence of Respondents	**Number**
City	716
Countryside	403

Note: Source: Results of own research.

Table 2 Examined Chocolate Yoghurts and Their Designation.

Sample Designation	**Examined Yoghurt**
Sample 1	Billa živá kultúra
Sample 2	Agro Tami živý jogurt
Sample 3	Billa bezéčkový jogurt
Sample 4	Bánovecký bezéčkový jogurt
Sample 5	Z láska k tradícii
Sample 6	Zvolenský smotanový jogurt
Sample 7	Lidl smotanový jogurt

Note: Source: Results of own research.

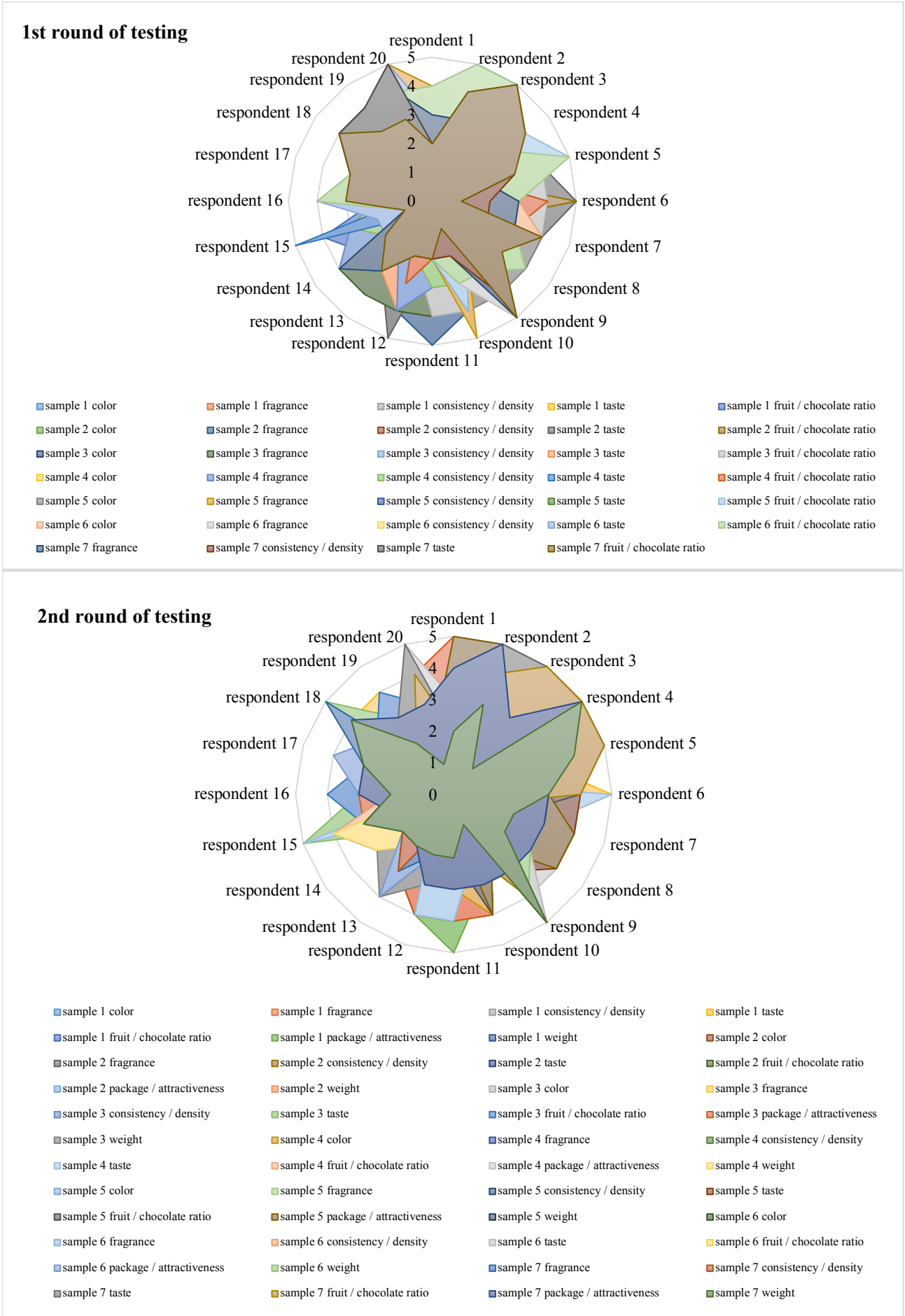

Figure 3 Comparison of Results of Two Rounds of Blind Testing. Note: Source: Results of own research.

The aim of this research, as it has been mentioned before, was to find out whether the packaging of a given product affects the consumer's purchasing decision. Respondents were tasked on a scale of 1 to 5, with 1 being the best rating and 5 the worst to evaluate selected attributes of the examined yogurts. These attributes were color in the first round of testing; aroma; consistency, resp. yogurt density; flavor and proportion of chocolate. In the second round of testing, the package size (i.e. whether it is suitable for respondents) and the attractiveness of the package was added to the attributes.

The results of both rounds of mentioned blind test point to many interesting findings – while in the first round of the blind test carried out in the overall evaluation of the respondents, as the best yogurt was evaluated the yogurt sample No. 5, in the second round there were the samples No. 7 and No. 6 (Figure 3).

If we look at the evaluation of the yogurt in more detail, we would find that while in the first round, when comparing samples No. 1 and No. 2 representing identical yogurt, the sample No. 1 was evaluated better, where respondents also perceived higher proportion of chocolate, even better taste of the yogurt. When comparing samples No. 3 and No. 4, representing identical yogurts than sample No. 4 was evaluated better only in color, the other attributes were perceived and evaluated in both samples the same way, except the taste, that was slightly better in Sample No. 3 and the smell that was better in Sample 4. In a comparison of samples No. 5, No. 6 and No. 7, respondents reported very similar ratings but based on the share of chocolate as the worst were evaluated the samples No. 6 and No. 7. In the second round of blind testing, when the respondents knew the specific yogurts and therefore could be influenced not only by the packaging but also a specific brand of yogurt, was in case of samples No. 1 and No. 2 better-evaluated sample No. 1 regarding the volume of packaging and sample No. 2 regarding the taste. When comparing the samples No. 3 and No. 4, sample No. 3 was better rated in terms of color and sample No. 4 in terms of taste and volume of the packaging; and when comparing samples No. 5, No. 6 and No. 7, the best rated for the attractiveness of packaging was sample No. 5, the best taste was rated in sample No. 6, and the best smell in sample No. 7. Consistency was evaluated as the best one in sample No. 6 and No. 7.

In terms of evaluation of packaging attractiveness and the impact of packaging on consumer behavior can be said that in this case, irrationality prevails, as already mentioned, identical yogurts were examined in the blind test but under different brands but respondents felt differences in taste, consistency, etc. Thus, the evidence indicates that the packaging can influence the consumer's decision and plays an important role in his purchasing decision and the evaluation of the en product.

The most attractive packaging in the case of our research was considered packaging with traditional "Slovak" motives and figures.

The last questions of the questionnaire survey focused mainly on factors leading and discouraging to and from the purchase of private label products, resp. what would affect our respondents to buy the products the most? Based on the evaluation of these questions, it can be said that while the most important factors leading to the purchase of private label products are *the combination of reasonable price and quality* (47% of respondents), *perceived quality* (13.2% of respondents) and *good experience* (11.4% of respondents) and not the price by itself how it was until recently **(Burt, 2000; Kumar and Steenkamp, 2007)**. The most important factors discouraging the purchase of private label products are *the expected low quality* (17.2% of respondents), *nonacquaintance of the producer* (16.8% of respondents), and *freshness* (14.9% of respondents).

Again, in terms of our respondents' purchase of private label products can be seen many interesting findings – as consumers are in an increasingly competitive and dynamic market environment in today's modern and globalized world **(Smutka et al., 2016; Polakevičová, 2015; Džupina, Hodinková and Kiková, 2016; Lorincová et al., 2018; Ližbetinová et al., 2019; Mach, Dvořák and Hošková, 2018)** characterized mainly by the online environment and the supersaturation of traditional communication tools, online communication, digital marketing, influencer marketing, etc. However, this assumption has not been confirmed as our respondents prefer more traditional forms of marketing communication, such as *word of mouth marketing and friends´ recommendations* (40.3% of respondents), *more interesting form of promotion* (15.9% of respondents), *tasting* (15% of respondents) *and free samples or buy 1 get 1 free* (14.8% of respondents).

Evaluation of tested dependencies

Hypothesis 1: We assume there is a correlation between the quality rating of private label products and the gender of respondents –confirmed.

Hypothesis 2: We assume there is a correlation between the purchase of private label products and the gender of respondents –confirmed.

Hypothesis 3: We assume there is a correlation between the purchase of private label products and the economic activity of respondents – rejected.

Hypothesis 4: We assume there is a correlation between the perception of private label packaging and the gender of respondents – rejected.

Hypothesis 5: We assume there is a correlation between the perception of facts that influence respondents to buy the private label products and the gender of respondents – rejected.

Hypothesis 6: We assume there is a correlation between the perception of facts that influence respondents to buy the private label products and the age of respondents – rejected.

Hypothesis 7: We assume there is a correlation between the decisive factor when buying private label products and the gender of respondents – confirmed.

Hypothesis 8: We assume there is a correlation between the decisive factor when buying private label products and the age of respondents – rejected.

Hypothesis 9: We assume there is a correlation between the facts that discourage respondents from buying private labels and the gender of respondents – confirmed.

Hypothesis 10: We assume there is a correlation between the facts that discourage respondents from

buying private labels and the age of respondents – confirmed.

CONCLUSION

Even though private labels are not a complete novelty in the market, they still represent a kind of uncharted territory that needs to be explored. The submitted paper dealt with the issue of private labels and their perception by Slovak consumers as well as with the influence of packaging and chosen marketing communication tools on consumer purchasing decisions in the dairy segment, as the results of several surveys proved that private labels are mainly purchased in this segment.

An anonymous questionnaire survey was chosen as the main research method, which was attended by total 1,116 respondents from all over Slovakia, and was subsequently supplemented by the blind test attended by total of 20 respondents aged up to 25 years. The results of our two researches have shown several interesting and key findings, which proved that while the reasons, resp. rationality prevails over irrational factors leading to and discouraging the purchase of private products, irrationality prevails in the perception and evaluation of packaging. As far as the marketing communication techniques and methods are concerned, based on the results of our research, it can be concluded that the "heart" rather than reason prevails, since it is still true that respondents prefer more traditional forms of marketing communication, such as *word of mouth marketing and friends´ recommendations, more interesting form of promotion, tasting and free samples or buy 1 get 1 free* and not the modern techniques and forms of addressing customers, which we have expected before.

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EFFECTS OF PROCESSING METHODS ON NUTRITIONAL COMPOSITION OF FISH *PAMPUS ARGENTEUS* (WHITE POMFRET) IN IRAN

Ali Aberoumand

ABSTRACT

The effects of different processing methods (Frying cooking, brining and boiling) on the proximate composition of fish species (*Pampus argenteus*) were investigated. The objective of this work is to know the best processing methods, the effect of processing on nutritional values of fish products. The result of the proximate composition of the fish species showed that the highest protein content (38.17%) was in *P. argenteus* processed with the frying process. The result of moisture content indicated that boiled samples were consistently the least (25.20%) while for fried sample had the highest moisture percentage. The lipid was reduced to the least value of 9.94% in the brined fish. In cooking, the important factors for consideration are moisture, lipids, and protein, though low moisture would ensure a fish product with extended shelf life. To have a longer shelf life, high protein is desirable, a low lipid is equally desirable as to reduce oxidation and rancidity in the samples which causes off-flavor and bad taste in fish products. In conclusion, all the processing methods are good and could extend the shelf life of the products with an exception of boiling method; they could keep the fish fillet free from spoilage and microorganisms attack for some period. This study showed that the proximate values obtained could be of help in choosing fish based on nutritional values.

Keywords: frying; brining; boiling; processing; *Pampus argenteus*

INTRODUCTION

Fish and seafood are a source of good quality protein which is essential for health **(Kim and Lall, 2000)**. Fish is usually cooked in different ways such as boiling, smoking, baking, frying, and grilling. These cooking methods result in enhancing flavor, taste, and improve the digestibility and inactivate the pathogenic microorganisms and enzymes **(Kocatepe et al., 2011)**.

During cooking of fish, some chemical and physical reactions take place such as protein denaturation that increases its digestibility and improves the nutritional value. However, the contents of fat-soluble vitamins or polyunsaturated fatty acids are often reduced **(Alizade et al., 2009)**.

Deep Fat Frying (DFF) is a major cooking method and is considered to be one of the oldest methods of food preparation. It is a cooking method of immersing foods in hot oil at a temperature above the boiling point of water. The oil temperature usually varies from 130 to 200 °C. During frying, there are many chemical reactions such as browning, gelatinization, and denaturation due to the elevated temperature of the product **(Tangduangdee, Bhumiratana, and Tia, 2003)**.

Pampus argenteus (White pomfret) is one of the most preferred indigenous aquaculture species in Iran due to better growth. Nutritional information is only available for fresh fish (Figure 1). The study was carried out to determine nutritional composition (protein, fat, ash, moisture, and energetic values) of brined, boiled, and fried *Pampus argenteus*.

These cooking methods result in enhancing organoleptic properties and improve the digestibility and inactivate the pathogenic microorganisms **(Kocatepe et al., 2011)**. Method cooking of fish, lead to some chemical and physical reactions take place such as protein denaturation that increases its digestibility and improves the nutritional value. Meanwhile, the contents of fat-soluble vitamins or polyunsaturated fatty acids are often reduced **(Alizade et al., 2009)**. Deep fat frying is a major cooking method and is mentioned as one of the old methods of food preparation. It is a cooking method of immersing foods in plant hot oil at a temperature above the boiling point of water. The oil temperature usually is around 180°C. During frying, there are many chemical reactions that take place such as browning, gelatinization, and denaturation due to the elevated temperature of the product **(Tangduangdee, et al. 2003)**.

Salting is the oldest, applied, and commonly used processing technique for fish preservation all over the world because of the simplicity of the process and low production cost **(Martínez-Alvarez and Gómez-Guillén, 2013)**. Salt is effective as a preservative compound because it reduces the

Figure 1 Fish *Pampus argenteus* (White pomfret).

water activity of fish fillets, consequently, microorganism growth and enzymatic spoilage are inhibited. Also, the aim of salted fish is to improve sensory properties more than preservation **(Mujaffar and Sankat, 2005)**.

Scientific hypothesis

The different processing methods will affect the composition and energetic values of the Pampus argenteus fish fillets.

MATERIAL AND METHODOLOGY

Raw materials

This study was carried out in the Department of Fisheries, Behbahan Khatam Alanbia University of Technology in November 2019. *Pampus argenteus* (White pomfret) fresh fish, similar sizes (54 – 66 cm) and weights (3.456 – 3.234 kg), were purchased from the fish market of the city of Behbahan, Iran, they were immediately stored in containers with ice and transported to the fish analysis laboratory of the Behbahan Khatam Alanbia University of Technology for processing and analysis.

Sample preparation and cooking methods

White pomfret fish were caught from the southern waters of Iran during September 2019. A homogeneous lot of fish was kept in a cold iced box and transported to the laboratory within 20 min. Samples were then filleted and then fish fillets were divided into 3 groups. The first group was uncooked (raw samples); the other 2 groups were cooked by different heat treatments (boiling and frying) and the third group was brine salted treated.

Boiling

The fillets were uniformly placed forming a thin layer on a stainless steel steamer above a stainless steel pot of boiling water and cooked with the lid on for 20 min.

Frying

The fillets were uniformly placed forming a thin layer in a wire mesh basket and immersed in Sunflower oil in a deep fryer for 10 min at 180 °C.

Brine salting

In the brine salting method, the cleaned fish were placed inside plastic jars of 5 liters, and brine was added until the fish was completely covered and brined in 30% salt solution, fish fillet to salt solution ratio is 1:1. were placed for 15 min in a special container **(Binici and Kaya, 2018)**. The producer and purity of chemicals used for experiments were according to a standard method by **Binici and Kaya (2018)**.

Analytical procedures

The heat treatment methods were performed in triplicate. After all heat treatments, the samples were cooled to 25 °C temperature. Each sample of raw or cooked fish fillets was crushed using a kitchen blender (made in Iran) and the fish powder was used to determine proximate chemical composition.

Proximate chemical composition

The moisture content of cooked and uncooked fish fillets was dehydrated by an oven at 115 °C until a constant weight was obtained **(AOAC, 2005)**. Crude protein content was calculated by converting the nitrogen content determined by Kjeldahl's method (6.25 × N). Fat content was determined according to the Soxhlet method **(AOAC, 2005)** by using chloroform as an extraction solvent. Ash content was determined by incineration in a muffle furnace (Made in Iran) at 600 °C for 2.5 h **(AOAC, 2005)**. The total caloric value was calculated from the corresponding caloric coefficients for proteins, lipids, and carbohydrates, respectively 4, 9, and 4 kcal.g^{-1} **(USDA, 2015)**.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 16.0. Software for Difference in the means between the groups was analyzed using the T-test. Duncan's multiple range tests were applied to do multiple means comparison. Statistical significance was set at $p < 0.05$.

Table 1 Proximate composition (g.100g^{-1}) and caloric value of fresh,brined, fried and boiled fish *Pampus argenteus*, on a dry basis.

	Moisture	Protein	Lipids	Ash	Calories (Kcal.100g^{-1})
Fresh	24.60 ±0.28^{a}	13.60 ±0.38^{a}	55.80 ±0.23^{a}	6.00 ±0.07^{a}	556.60 ±1.9^{a}
Boiled	25.20 ±0.4^{b}	29.40 ±0.97^{b}	38.40 ±0.55^{b}	7.00 ±0.12^{b}	463.2 ±1.61^{b}
Brined	38.80	33.56	9.94	17.70	223.6
Fried	40.40	38.17	12.23	9.20	262.75

Note: Mean of samples analyzed in duplicate. The same letters in the column do not differ from each other at the 5% level of significance.

RESULTS AND DISCUSSION

Results for proximate composition analysis (protein, fat, moisture, and ash) for fresh, boiled, fried, and brined fish *Pampus argenteus* are presented in Table 1.

Determined protein content for *P. argenteus* was 13.60%, 29.40%, 38.17%, and 33.56% for fresh, boiled, fried, and brined fish respectively (Table 1). Protein content was significantly increased (p <0.05) is processed (boiled, fried, and brined) fish. Lowest protein levels were nevertheless observed in boiled fish. The highest and lowest levels of fat were recorded in brined (9.94%) fish and were significantly different from unprocessed (fresh) fish. Brined and fried fish had significantly high levels of ash (17.70% and 9.20%) (p <0.05). There were significant differences (p <0.05) in moisture content for *P. argenteus* where the lowest levels were recorded in boiled fish (25.20%).

Chemical composition of fish varies widely depending on the fish species, age, nutrition, size and sex, growth place, catching season, and the environmental conditions **(Manthey, Karnop and Rehbein, 1988)**. The increase in the fat content of the fried fish fillets is related to oil absorption during the cooking process. Also, reported that the increase in dry matter content was observed in the fried and grilled fish fillets. The decreased moisture content was noticed in all the cooking methods except for the boiled fillets. Increased ash content was noticed in all the cooked fillets when compared to raw fish fillets. That deep-fried fish had the highest protein value comparing other cooking methods. Water loss, occurring during cooking resulted in increasing protein content in the fried fish samples **(Gökçe et al., 2004; Gokoglu, Yerlikaya, and Cengiz 2004)**. The higher lipid content of fried fish than other methods is mainly due to the absorption of oil by the fish and losing moisture during frying process (the apparent higher ash content of a fried sample is due to more loss of moisture took place during deep-frying cooking comparing with another method **(Küçükgülmez et al., 2006))**. A higher level of protein in this study agrees with earlier reports **(Saguy and Dana, 2003)**.

Cooking methods that require heat can affect the nutritional composition of fish depending on the intrinsic composition, temperature, and time the product is exposed and the method used. The water loss may be attributed to the evaporation, dehydration of the fillet fibrils, and probably to some heat-induced protein denaturation during boiling, which causes less water to be entrapped within the protein structures **(Chukwu and Shaba, 2009; Costa et al., 2013)**.

Quality properties of processed fish at the time of consumption and eating greatly depends on different factors such as; the freshness and fish quality, the cooking methods, and the storage or preservation conditions followed before cooking. Fish spoilage is usually more rapidly than other animal muscle foods and this spoilage is primarily bacterial. Therefore, good preservation techniques must prevent the bacterial spoilage of fish without affecting its quality and nutritional value **(El-Lahamy et al., 2018)**.

Marimuthu et al. (2012) showed that the increase in dry matter content was observed in fried fish fillets. The highest moisture content (77.2%) was recorded in raw fillets. Increased ash content was noticed in all the cooked fillets when compared to raw fish fillets. Moisture loss was also found in baked fillets of snakehead fish. However, the dehydration rate comparatively was lower than during frying. These changes were similar to those reported by **Gokoglu, Yerlikaya and Cengiz (2004)** in rainbow trout and **García-Arias et al. (2003)** in sardines. Water losses, occurring during frying resulted in higher protein content in fried fish as compared to the raw fish fillets **(García-Arias et al. 2003)**. Accordingly, the increase in ash, protein, and fat content found in cooked silver catfish fillets is explained by the reduction in moisture. These results were similar to our present study.

Abraha et al. (2018) reported that generally fish processing methods (high and low-temperature treatments) including, chilling, freezing, canning, smoking, drying, salting and frying, and various combinations of these, to give the fish product a form which is attractive, fresh to the consumers and prolong and suitable storage life. These processing methods have different applications, techniques, and significant influences and effects on the chemical, physical, and nutritional composition of processed fish. This is because heating and exposure to a high concentration of salt lead to chemical and physical changes. Ultimately different quality could be obtained via these methods, hence subsequent effects on processed fish's shelf life also vary **(Magnussen et al., 2008; Díaz-Tenorio, García-Carreño and Pacheco-Aguilar, 2007)**.

CONCLUSION

The results obtained from this study showed that the highest protein (38.17%) and moisture (40.40%) contents found for fried fish *Pampus argenteus*, while lowest moisture content (25.20%) found for boiled fish. The least lipid content (9.94%) found for brined fish (p <0.05). Fish provides the most dietary animal valuable protein to people in Iran. Findings also confirm earlier reports that processing alters nutrients content in fish. Although fried fish are mostly liked by many people, results in this study have demonstrated that more nutritional benefits could be obtained when fish are processed through the boiling method.

Lower amounts of lipids in boiled samples were presumably the result of the spread of fat into stock during boiling. As far as fat is concerned, the loss introduced by heat treatment is not as explicit as in the case of water. Boiling, both with and without the addition of salt and, caused a substantial (10% on average) drop in the amount of fat. The loss of fat and nutrients in boiling was less than frying in oil. It has been shown also that boiled fish exhibit better storage properties due to low moisture retention

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FOOD ADULTERATION AND SAFETY REGARDING DETECTED MARKET CASES AND CONSUMER OPINIONS

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ABSTRACT
Food fraud is one of the long-standing causes of scandals attracting particular attention for a long time. This study aimed to monitor food fraud in the European Union and to identify the relationships among the countries where the cases were reported, adulterated commodities (seafood, eggs, milk, meat, fish, and their products) and types of fraud. The secondary data were covered by the survey focused on consumer knowledge about fraudulent activities, ingredient substitution, masking of origin, mislabeling, placing on the market of foods not fit for human consumption within Slovak inhabitants. Primary and secondary data were used to achieve this aim. Primary data were obtained from the Food Fraud and Quality Knowledge Center (KCFFQ) and secondary data from the questionnaire survey from 354 respondents. During the period from 2017 to 2019, 163 cases of food fraud were reported, most of which originated from Italy and mainly concerned fish and fish products. Based on primary data and one-way ANOVA statistical tests, we confirmed five hypotheses. There was found no statistical impact of the country on the type of food fraud ($p = 0.0067$), but the significant effect was determined on which food was adulterated ($p = 0.000001$). There was no statistical correlation among years and countries where the cases were reported ($p = 0.110$), but the statistically significant correlation was confirmed among years and commodities ($p = 0.0043$) and types of fraud reported ($p = 0.009$). Based on the processed secondary data from the questionnaire, we can conclude some information or public interest in food fraud problems.

Keywords: food fraud; KCFFQ; consumers; adulteration; questionnaire

INTRODUCTION
Adulteration in food has been a concern since the beginning of civilization, as it not only decreases in the quality of food products but also results in several ill effects on health. Authenticity testing of food and adulterant detection of various food products is required for value assessment and to assure consumer protection against fraudulent activities. Concerns about food safety and regulation have ensured the development of various techniques such as physical, biochemical/immunological, and molecular techniques, for adulterations detection in food **(Bansal et al., 2017)**. Food fraud, the intentional misrepresentation of the true identity of a food product or ingredient for economic gain, is a threat to consumer confidence and public health and has received increased attention from both regulators and the food industry **(Everstine et al., 2018)**. Every European citizen has the right to know how the food he eats is produced, processed, packaged, labelled, and sold. The implementation of this integrated Food Safety policy in the EU involves various actions, namely to assure effective control systems and evaluate compliance with EU standards in the food safety and quality, animal health, animal welfare, animal nutrition and plant health sectors within the EU and in non-EU countries in relation to their exports to the EU; to manage international relations with non-EU countries and international organisations concerning food safety, animal health, animal welfare, animal nutrition, and plant health; to manage relations with the European Food Safety Authority (EFSA) and ensure science-based risk management **(European Commission, 2020a)**. Rapid alert system for the notification of a direct or indirect risk to human health deriving from food or feed was established in the EU. It involves the Member States, the Commission, and the EFSA **(Regulation (EC) No 178/2002)**. Starting from November 2015 a dedicated IT application known as the Administrative Assistance and Cooperation System (AAC) has been made available for the Member States. After a successful period of testing dealing with fraudulent practices in the agri-food chain, in 2016 the system was also opened to liaison bodies based on official controls. The AAC and RASFF (Rapid Alert System for Food and Feed) are working together in synergy to keep the high EU standards for food and feed **(EU, 2017; RASFF, 2018)**.

The Knowledge Centre for Food Fraud and Quality provides and shares up-to-date scientific knowledge on food fraud and food quality issues. It coordinates market surveillance activities and operates early warning and information system for food fraud. Collectively is operated by the European Commission's science and knowledge

service, the Joint Research Centre (JRC), and the Departments regulating the feed-food chain and protecting consumer rights. The Centre complements the activities of the EU Food Fraud Network, which is operated by the European Commission Department for Health and Food Safety **(European Commission, 2020b)**.

Scientific hypothesis

The first objective of this study was to monitor the food fraud focused mainly on the food of animal origin in member states of the European Union and to identify the most common reasons for these cases. The following hypotheses concerning food fraud were set up as follows:
Hypothesis 1: We assume no statistical evidence of the country's impact on food frauds, which were reported.
Hypothesis 2: We assume statistical evidence of the country's impact on counterfeit foodstuff.
Hypothesis 3: We assume no statistical evidence between the countries and year when the food fraud cases were reported.
Hypothesis 4: We assume statistical evidence between adulteration of food and the year of its occurrence.
Hypothesis 5: We assume statistical evidence between the type of food fraud and year of its occurrence.

The second objective was to find out consumer awareness in Slovakia regarding their knowledge and opinion on food fraud.

MATERIAL AND METHODOLOGY

The objective of this paper was achieved by using and processing of primary and secondary data. Primary data were obtained from the Knowledge Centre for Food Fraud and Quality (KCFFQ) which is hosted by the Joint Research Centre (JRC), during the period 2017 – 2019. The records were obtained using the portal contained information about:

- the reporting country,
- the food commodities,
- categories of food fraud,
- accurate case information.

The data obtained became the basis for confirming or rejecting hypotheses.

Secondary data were obtained using the survey focused on identifying consumers' awareness of food fraud in the Slovak Republic. The questionnaire was performed at a sample of 354 respondents in the 2019 year, using the Google Forms platform. The respondents were diversified into 2 categories in terms of gender and age. Women were represented by the amount of 278 (78.5%). The men were represented by 76 respondents (21.5%). Group was also divided into four groups based on their age. The age structure consisted as follows: from 16 to 21 years (13.3%), from 22 to 30 years (41.2%), from 31 to 45 years it was 27.1% and from 46 to 70 it was 18.4%. Questions about which commodities are most often adulterated, which types of food fraud occur most often, in which countries they think food fraud is the most commonly reported, and from what media they get this information was given to respondents.

Statistical analysis

The collected data were processed using the statistical program XLSTAT (Addinsoft, version 2019.1.2) The formulated hypotheses were tested using the one-way ANOVA statistical test. Hypotheses were tested: if the *p*-value is less than a significant level (0.05) the invalid hypothesis was rejected and an alternative hypothesis was confirmed.

RESULTS AND DISCUSSION

Analyzes of primary data from KCFFQ

Food safety is one of the crucial issues of public health protection **(Cieslik and Cieslik, 2012)**. If food is misdescribed, i.e. the information about the origin, composition, etc. provided to customers is not true and if this misdescription is done to deceive the customer for financial gain, food fraud, also known as economically motivated adulteration, is committed. Economically motivated adulteration of food is estimated to cause damage of around €8 to €12 billion per year **(Vaqué and Vidreras, 2018)**.

By evaluating food fraud notifications at KCFFQ during years from 2017 to 2019, we found 163 notifications reported to the KCFFQ system (Table 1a, 1b, 1c, 1d, 1e, and 1f). We evaluated the ten most common types of fraud, 12 reporting countries, 5 commodities that were most commonly adulterated. There were found 60 notifications in 2017, where in Slovakia was reported only one case. This notification involved meat originating from Brazil contaminated with *Salmonella*.

The next year 2018, showed the highest amount of notifications for the whole observed period, namely 72.
In 2019 there were found 31 reports. In Slovakia, no notification of food fraud was reported in the year 2018 and 2019. Reports of food fraud in each country from 2017 to 2019 in Europe are shown in Figure 1. Within this period, 92 reports were received from Italy, of which 37 concerned fish and fish products. Spain received 25 reports, also related to fish and fish products. 12 reports originated from the UK and 8 of them related to meat and meat products. 7 reports were reported from Belgium and Portugal, in both countries 6 cases related to meat and meat products, together 6 cases originated from France and 4 related to fish and fish products. 5 cases were reported from the Netherlands with 3 concerning the eggs. 3 cases from Ireland related to meat and meat products, Malta, Poland, and Slovakia reported one case of meat fraud and Germany reported one case of egg fraud.

Čapla et al. (2019) published a review on different types of foreign matter detected in food, reported by the RASFF during the period from 2016 to 2018. The presence of foreign bodies in food from different European regions showed differences. Plastic, glass, and metal were the most commonly reported in Western Europe, pests, and rubber in Northern Europe. As far as food commodities are concerned, bakery and confectionery products, fruit and vegetables, and convenience foods were the most frequently reported and the notifications originated often from Western Europe. Notifications from this part of Europe were made concerning other monitored commodities as well. Regarding the notification type, the most frequent one was an alert, and, in the case of a risk decision, serious risk constituted the largest part. Following updates to food safety certification standards and publication of new U.S. regulatory requirements, **Everstine et al. (2018)** undertook

a project to develop a scheme to classify food fraud-related adulterants based on their potential health hazard and apply this scheme to the adulterants listed in a database of 2,970 food fraud records. The classification scheme was developed by a panel of experts in food safety and toxicology from the food industry, academia, and the U.S. Food and Drug Administration. Results reinforce the importance of including a consideration of food fraud-related adulterants in food safety systems.

Regarding the particular problems found in our evaluation, we can see several kinds of foods reported as the problem with traceability (from Italy, Spain, Portugal, France, etc.). For example, Italy reported at 3.5 mil. eggs, that they were found to be untraceable.

Table 1a An overview of food frauds in 2017 – 2019 in the order in which they were reported.

Country	Commodity	Food fraud	Details
2017			
Italy	fish and fish products	mislabelling	incorrect labeling of fish during the Christmas period
Italy	fish and fish products	mislabelling	fish were not traceable
Italy	fish and fish products	artificial enhancement	dyeing low quality tuna to sell it as fresh fish
Italy	milk and milk products	counterfeit	counterfeiting of mozzarella (discovery of labels of a well-known dairy company stored along with cow milk from other companies)
UK	meat and meat products	substitution	selling turkey instead of halal lamb
Italy	milk and milk products	artificial enhancement	spoilt milk was treated with caustic soda to mask acidification and aging
Italy	milk and milk products	Substitution	replacement of buffalo milk for cow's milk
Italy	milk and milk products	artificial enhancement	smoked Provolone cheese with cardboard and printed and glued paper leaflets.
Spain	seafood	mislabelling	no records of octopus traceability
UK	meat and meat products	origin masking	claimed their lamb products had been 'Produced in Britain' but in fact contained traces of imported New Zealand meat
Italy	meat and meat products	origin masking	using inappropriate animals to produce the cured ham and forging documents to reconstruct the traceability of the meat
Netherland	meat and meat products	substitution	sold horse meat as beef
Netherland	fish and fish products	substitution	endangered shark found in several fish products
Italy	eggs	origin masking	declaration of origin and other labelling information were missing
Italy	fish and fish products	mislabelling	lack of or incomplete informations about traceability
Italy	fish and fish products	mislabelling	untraceable fish products
Italy	seafood	mislabelling	untraceable seafood products
Spain	fish and fish products	mislabelling	lack of traceability and proper labelling
Slovakia	meat and meat products	products not fit for consumption	meat coming from Brazil contaminated with *Salmonella*
Portugal	milk and milk products	mislabelling	untraceable fozen goat milk
Italy	fish and fish products	mislabelling	mislabelling and traceability problem
Italy	eggs	mislabelling	absence of labelling, making it impossible to determine the origin of the eggs
Italy	fish and fish products	mislabelling	mislabelling juvenile fishe
Italy	fish and fish products	illegal equipment	use of illegal equipment and catch of juvenile fishe
Spain	fish and fish products	mislabelling	lack informations or mislabelling of origin
Italy	milk and milk products	origin masking	doubts about the true origin of dairy products
Italy	meat and meat products	substitution	sale of pork sausages instead of advertised wild boar and deer sausages
Italy	fish and fish products	substitution	red tuna was substituted by Yellowfin tuna and grouper was replaced by cheaper Nile perch in restaurant
Spain	meat and meat products	substitution	adding pig meat, soy and bread to their beef burgers and meatballs

Table 1b An overview of food frauds in 2017 – 2019 in the order in which they were reported.

Country	Commodity	Food fraud	Details
2017			
Portugal	meat and meat products	products not fit for consumption	lack of veterinary controls; lack of traceability; expired food relabelled with a new expiry date
Italy	fish and fish products	mislabelling	mislabelling of fish
Italy	fish and fish products	substitution	bluefin tuna was sold as a cheaper Albacore fish
Spain	meat and meat products	products not fit for consumption	distributing horse meat (horses were too old or labelled as "unfit for consumption")
Belgium	meat and meat products	products not fit for consumption	distributing horse meat (horses were too old or labelled as "unfit for consumption")
Italy	food	mislabelling	selling frozen food in restaurant without properly informations on the menu
Italy	fish and fish products	products not fit for consumption	expired and/or unlabelled products
Italy	eggs	mislabelling	traceability problems
UK	meat and meat products	substitution	horse meat and regular beef were sold as 100% beef
Italy	eggs	contamination	high levels of fipronil detected
Netherland	eggs	contamination	high levels of fipronil detected
Belgium	eggs	contamination	high levels of fipronil detected
Germany	eggs	contamination	high levels of fipronil detected
United Kingdom	fish and fish products	mislabelling	labels of other companies were used to by-pass the Russian law limiting the number of UK exporters
Italy	milk and milk products	origin masking	cheese were produced in unregistered places, labelled with incorrect information and entered fraudulently the Fontina DOP production chain
Italy	fish and fish products	mislabelling	unlabelled fish products
Italy	milk and milk products	origin masking	dairy farms were not accredited for the production of cheese with a protected origin and re-used old labels to pass the product as authentic
Spain	fish and fish products	artificial enhancement	the nitrites contained in the vegetables give the tuna a bright red colour making it appear fresher
Italy	milk and milk products	origin masking	labelling rules concerning claims made on the geographical origin and/or being organic were falsed claims
Italy	fish and fish products	mislabelling	unlabelled fish and molluscs
Italy	food	mislabelling	food without any traceability
Italy	fish and fish products	mislabelling	problems of traceability and compliance to mandatory labelling
Spain	fish and fish products	mislabelling	untraceable and unlabelled tuna
Italy	meat and meat products	mislabelling	of poor hygiene and non-traceability distibuted to schools, hospitals and military canteens
Italy	eggs	mislabelling	3.5 million eggs were found to be untraceable
Italy	fish and fish products	mislabelling	fish were found with irregularities concerning the labelling and traceability
Spain	fish and fish products	artificial enhancement	dyeing low quality tuna to sell it as fresh fish
Italy	fish and fish products	mislabelling	mislabelling of fish
Italy	fish and fish products	substitution	Bluefin tuna was sold as a cheaper Albacore fish
Italy	fish and fish products	mislabelling	health inspection, origin, date of freezing and traceability documentation were missing
Italy	milk and milk products	mislabelling	untraceable products in the food chain

Table 1c An overview of food frauds in 2017 – 2019 in the order in which they were reported.

Country	Commodity	Food fraud	Details
Spain	seafood	mislabelling	the origin of the product could not be determined as the mandatory labelling was missing
2018			
Spain	fish and fish products	mislabelling	fish that could not be traced
Spain	fish and fish products	substitution	restaurants were selling other types than those on offer (replaced by sole, hake and bluefin tuna)
Italy	fish and fish products	mislabelling	sale of frozen foodstuffs as fresh food of incorrect Italian origin
Italy	meat and meat products	origin masking	sale of frozen foodstuffs as fresh food of incorrect Italian origin
United Kingdom	meat and meat products	substitution	lamb meat (animal <12 months) has been replaced by mutton meat (older animal)
Italy	fish and fish products	origin masking	the shellfish was not traceable and the documentation of origin was missing
Italy	milk and milk products	mislabelling	Untraceable food
Italy	fish and fish products	mislabelling	Untraceable food
Belgium	meat and meat products	mislabelling	minced meat witch the expiry dates of the products were falsified
Spain	seafood	origin masking	Portugal clams were sold as Galician clams
Belgium	meat and meat products	substitution	conventional meat were sold as organic meat
Italy	food	origin masking	the traceability documentation were missing
Spain	meat and meat products	products not fit for consumption	freezing products; adding warm water, viscera and pork blood to cows to increase body weight; the product has expired 3 years ago and products contaminated with S*almonella*
Italy	meat and meat products	origin masking	improper marketing of meat with registered trade mark
Ireland	meat and meat products	products not fit for consumption	horse meat imported from eastern countries were unfit for human consumption; sold without true evidence on the Irish market
Italy	fish and fish products	products not fit for consumption	distribution of expired ten tonnes of frozen fish
Italy	meat and meat products	counterfeit	Danish Duroc boars have been used instead of Italian pig breeds for insemination purposes to reduce the fat content of the hams produced
Italy	fish and fish products	mislabelling	lack of appropriate traceability documentation for tuna, salmon and other types of fish
Italy	fish and fish products	mislabelling	lack of appropriate traceability documentation for fish products
Italy	fish and fish products	products not fit for consumption	use low quality fish to prepare sushi and sashimi; offered frozen fish that have expired
France	fish and fish products	products not fit for consumption	fillets were sold as fresh fish, although they were actually frozen
France	fish and fish products	artificial enhancement	use of unauthorized additives to improve the red color of tuna
France	fish and fish products	artificial enhancement	salt, potassium lactate, potassium acetate, citric acid and polyphosphate were used to retaining water
Italy	milk and milk products	mislabelling	the product was not made with PDO cheese from Bergamo as indicated on the label
Belgium	meat and meat products	products not fit for consumption	poultry meat with a counterfeit expiry date were sold
Italy	meat and meat products	origin masking	counterfeiting ham with PDO San Daniele

Table 1d An overview of food frauds in 2017 – 2019 in the order in which they were reported.

Country	Commodity	Food fraud	Details
United Kingdom	eggs	mislabelling	the sale of eggs at high prices whilst they were free range eggs that did not actually meet the criteria
Italy	milk and milk products	substitution	false mozzarella di bufala DOP and other type of cheese were sold to tourists
Spain	meat and meat products	products not fit for consumption	ham and other types of meat that were expired, were re-labelled and re-introduced into the market
Italy	food	substitution	substitution of organic products by conventionally produced products; lack of hygiene and traceability of the products
Italy	food	products not fit for consumption	rotten foods, in some cases after the expiry date was relabelled and intended to be offered for sale
Spain	meat and meat products	mislabelling	the hams in the packages were sold with a changed expiration date or were rotten
Italy	fish and fish products	products not fit for consumption	defrosted fish placed on the market from a fishing shop that did not meet the hygiene conditions laid down by law were seized
Portugal	meat and meat products	products not fit for consumption	found rotten meat stored under unsuitable conditions
Italy	fish and fish products	products not fit for consumption	part of the fish was supposed to be sold fresh, but in fact it was rotten
Spain	fish and fish products	smuggling	endangered Spanish eel species smuggled into Japan via China
Italy	fish and fish products	mislabelling	fish were found without traceability documents
Spain	fish and fish products	artificial enhancement	fish that was caught by boats not equipped with the appropriate freezers were treated with additives to mimic the appearance of fresh fish
Spain	fish and fish products	counterfeit	canned tuna were sold as fresh tuna
Italy	fish and fish products	products not fit for consumption	fish in a restaurant was not fit for human consumption
Italy	food	mislabelling	mislabeling and missing food traceability documents
United Kingdom	meat and meat products	substitution	meat products were sold and produced from unspecified meat (the species was not mentioned on the label)
Italy	food	products not fit for consumption	meat and fish unfit for human consumption were sold in a restaurant
Italy	meat and meat products	counterfeit	ham was not produced according to the mandatory compliance process to be labelled as "Crudo di Parma"
Italy	fish and fish products	products not fit for consumption	non-compliance with hygiene regulations for the storage and handling of fish in markets and restaurants
Italy	milk and milk products	counterfeit	cheese sold with the false labels of the Pecorino Crotonese PDO cheese
Ireland	meat and meat products	mislabelling	minced meat without proper labelling
Ireland	meat and meat products	substitution	meat from species not declared on the label. Lamb was most frequently replaced by meat from other spices and cow was the most commonly undeclared species
United Kingdom	meat and meat products	mislabelling	selling meat labelled as the "Best of British" when actually it was sourced from abroad.
Italy	fish and fish products	mislabelling	found 500 kg of untraceable fish
United Kingdom	meat and meat products	substitution	selling beef as lamb in restaurant

Table 1e An overview of food frauds in 2017 – 2019 in the order in which they were reported.

Country	Commodity	Food fraud	Details
Italy	fish and fish products	mislabelling	restaurants were indicted for selling frozen fish as fresh.
Spain	meat and meat products	substitution	restaurants in Madrid did not correspond to the species indicated on the menu
Italy	fish and fish products	mislabelling	lack of traceability information
Italy	fish and fish products	products not fit for consumption	non-respect of the expiry date and lack of adequate storage conditions
Italy	milk and milk products	substitution	mozzarella, sold in Spain and claimed to be produced using buffalo milk was made mostly with cow milk
Belgium	meat and meat products	mislabelling	meat sold in Belgium under the quality mark Belbeef lacks traceability
Italy	food	substitution	conventional food were labelled as "organic"; in some fields the company produced food respecting the rules of organic production, while on other fields food was produced with the use of pesticides, herbicides, etc.
Italy	milk and milk products	counterfeit	"Grana Padano" and "Parmiggiano" were sold in Canada making fraudulent use of the label "made in Italy".
Italy	milk and milk products	substitution	sale of false mozzarella di bufala DOP
Italy	milk and milk products	substitution	mixing cow and buffalo milk to produce the popular Italian cheese and sold it under the label "mozzarella di bufala".
Italy	meat and meat products	origin masking	sold meat labelled as Italian which rather was the meat of animals grown in other countries, mostly Spain and France
Italy	fish and fish products	artificial enhancement	a large amount of bleach was found in a food production plant
Italy	seafood	products not fit for consumption	octopus, sepia and squid with expiry dates in 2010 were withdrawn from the market
2019			
Malta	meat and meat products	origin masking	Imported live animals were slaughtered but labelled as originating from Malta
Poland	meat and meat products	products not fit for consumption	captured meat from sick cows killed in a Polish slaughterhouse which was not fit for human consumption
United Kingdom	fish and fish products	substitution	fish and chips restaurants sold protected spiny dogfish and some other protected types of shark, which commercialization is forbidden in Europe
Netherland	eggs	products not fit for consumption	eggs contaminated with fipronil
Netherland	eggs	mislabelling	according to the labels the eggs came from a farm that produced "free range" eggs but this information turned out to be false
Italy	eggs	mislabelling	missing traceability information of 4 000 g eggs
Spain	meat and meat products	origin masking	hams which were not designated as PDOs were referred to as PDO hams
Spain	fish and fish products	mislabelling	sale of canned tuna as fresh tuna
Italy	eggs	mislabelling	sale of food commodities fraudulently labeled as organic
Italy	fish and fish products	mislabelling	selling cheaper fish at a much more expensive price to restaurants
Italy	eggs	mislabelling	lacking traceability information

Table 1f An overview of food frauds in 2017 – 2019 in the order in which they were reported.

Italy	food	origin masking	food was labelled as Italian but it came from another country
Italy	food	products not fit for consumption	food that did not meet the required hygienic conditions was on offer
United Kingdom	milk and milk products	substitution	buffalo mozzarella sold in restaurants and supermarkets, was made wholly or partly from cow's milk
Italy	milk and milk products	counterfeit	cheese was labelled as a Protected Geographical Indication (PGI) product without fulfilling the requirements; counterfeited cheese was found
Italy	milk and milk products	products not fit for consumption	the cheese was found after a few months, stored under inappropriate conditions
France	meat and meat products	substitution	meat patties made in Poland contained fat, skin, starch and soya which are not allowed for this type of product
Italy	eggs	products not fit for consumption	the eggs had an extended expiration date in the pack-house
Italy	meat and meat products	mislabelling	hams do not fulfil the requirements to bear the PDO label
Italy	meat and meat products	substitution	hams did not comply with the requirements to bear the PDO labels
Italy	seafood	mislabelling	lacked appropriate information to trace the product
United Kingdom	meat and meat products	mislabelling	some species were declared on the label but in reality were not present in the product, for instance, ham without pork
Belgium	meat and meat products	substitution	meat conventionally produced, and originating from the Netherlands was labelled and sold in Belgium as organic meat
Spain	meat and meat products	products not fit for consumption	meat was contaminated with *Listeria*
Spain	meat and meat products	mislabelling	meat producer sold contaminated meat to a second company, which in turn sold it without indicating on the label the name of the producing company
France	meat and meat products	dilution	fraudulent increase of the weight of chicken meat with water
Spain	meat and meat products	origin masking	meat sold as lamb in Burgos is from other countries
Italy	eggs	mislabelling	organic eggs were from hens in cages
Italy	food	products not fit for consumption	found rotten fish not fit for human consumption
Italy	meat and meat products	counterfeit	butcher sold regular beef as Japanese Kobe beef
Portugal	meat and meat products	products not fit for consumption	meat not fit for human consumption was sold
Portugal	meat and meat products	mislabelling	products that lacked traceability information and did not fulfil administrative requirements
Portugal	meat and meat products	products not fit for consumption	meat was not stored at the right temperature
Portugal	meat and meat products	artificial enhancement	samples contained sulphite, a substance which addition to meat is forbidden
Italy	fish and fish products	mislabelling	fish did not fulfil the legal traceability requirements
Italy	food	mislabelling	some items had a PDO labelling although they did not fulfil the required criteria
France	meat and meat products	products not fit for consumption	rotten meat without traceability
Italy	fish and fish products	mislabelling	sale of food commodities fraudulently labeled as organic

Food or feed which is launched to the market shall be adequately labelled or identified to facilitate its traceability, through relevant documentation or information in accordance with the relevant requirements of more specific provisions.

By the **Regulation (EC) No 178/2002** it is the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing, and distribution.

In samples of eggs, fipronil contamination was reported from several countries. Fipronil is an insecticide of the phenylpyrazoles class and an active ingredient of one of the popular ectoparasiticide veterinary products. Fipronil is also formulated as insect bait for roaches, ants, and termites; as a spray for pets. In humans, poisoning is mainly due to accidental ingestions or suicidal attempts. In agriculture, fipronil is widely used for soil treatment, seed coating, and crop protection **(Ramesh and Milatovic, 2014)**. **EU Regulation (EC) 396/2005** set up the maximum levels for fipronil (0.005 $mg.kg^{-1}$ in chicken eggs) in several raw materials and foods. As consumers pay extra for perceived benefits of free-range eggs, this market sector presents profitable opportunities for producers with expertise in managing hens with enhanced behavioral freedom **(Newberry, 2017)**. According to the labels, some of our samples of eggs came from a farm that produced "free-range" eggs but this information turned out to be false. Several countries reported dyeing low-quality tuna to sell it as fresh fish or case of sulphite addition to meat. Unprocessed foods belong to the food in which the presence of a food dye may not be permitted **(Regulation EC no. 1333/2008)**. Conventional meat and other foods were sold as organic in several cases. Demand for organic meat is partially driven by consumer perceptions that organic foods are more nutritious than non-organic foods **(Średnicka-Tober et al., 2016)**.

The increasing consumer demand for organic products caused the organic food market has expanded in all continents of the world. Organic foods represent a specific segment of the food market **(Kozelová, Vietoris, and Fikselová, 2013b)**.

By our results found at the market, we can agree with the statement of **Vaqué and Vidreras (2018)** that common types of food fraud include the substitution of an ingredient with a similar, cheaper ingredient, the inclusion of undeclared ingredients, adulteration of foods to improve some of their characteristics; non-declaration or false declaration of processes and false declaration of the origin or geographic region of production of a food item.

There were reported several problems regarding the PDO label. EU quality policy aims at protecting the names of specific products to promote their unique characteristics, linked to their geographical origin as well as traditional know-how. By the **JRC Food Fraud Monthly Report (2019)** the inspectors that grant the Prosciutto di Parma and San Daniele PDO labels have resigned the irregularities that have recently been affecting the certification body responsible for the mentioned PDOs. In May 2019, inspectors revealed that 2.5 million hams did not comply with the requirements to bear the PDO labels.

Hypothesis 1 did not assume a link in the country's impact on food fraud. Based on the one-way ANOVA test, this hypothesis was confirmed (p = 0.0067). Hypothesis 2 assumed the influence of the country on which commodities were adulterated. Based on the one-way ANOVA test (, this hypothesis was confirmed (p = 0.000001). Hypothesis 3 did not assume the link among the countries and the years in which the cases were reported. Based on the result of the one-way ANOVA test, this hypothesis was confirmed (p = 0.110). Hypothesis 4 assumed that there was a link between foods that were adulterated and the years that occurred. Based on the result of the one-way ANOVA test, this hypothesis was confirmed (p = 0.0042). Hypothesis 5 assumed that there was a link between the type of counterfeiting and the years that occurred. Based on the result of the one-way ANOVA test, this hypothesis was confirmed (p = 0.009).

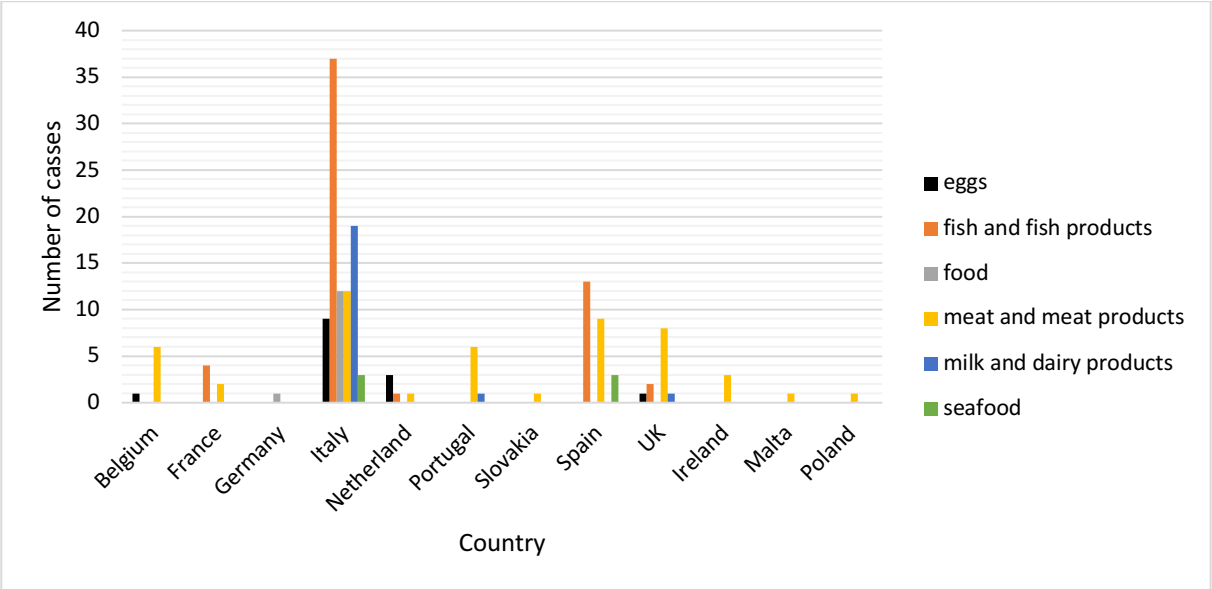

Figure 1 Country in Food Fraud Reports from 2017 to 2019 in Europe.

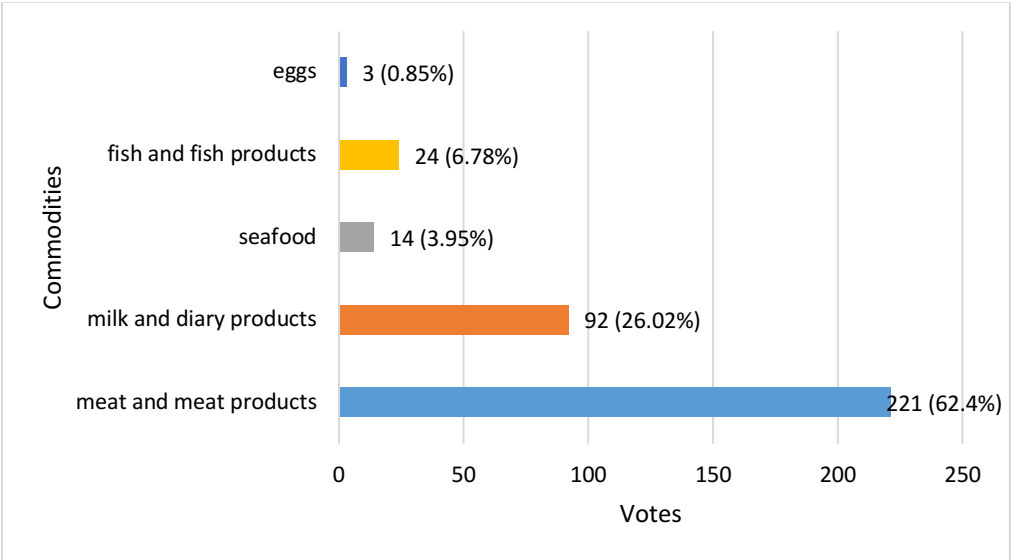

Figure 2 The answer to the question „which commodities are mostly adulterated?".

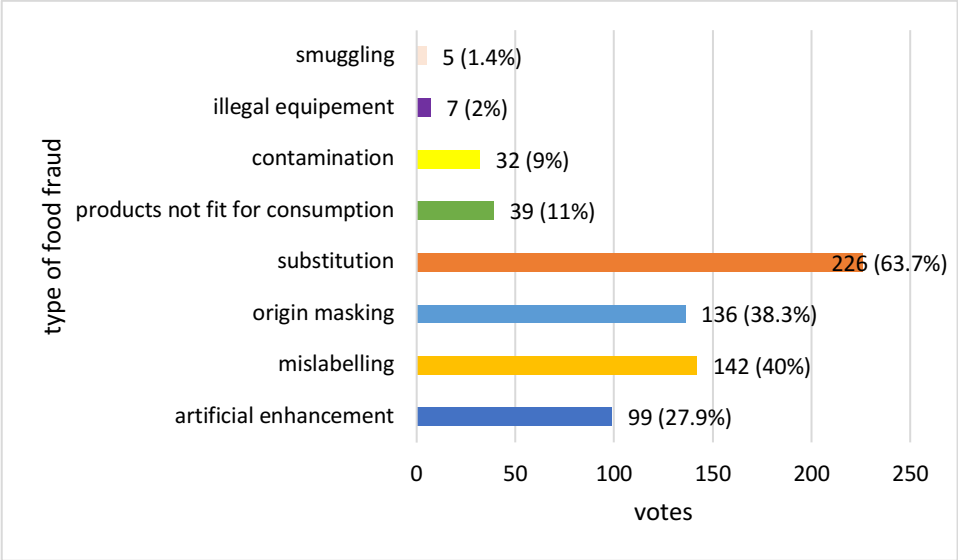

Figure 3 The answer to the question „which of the following causes of food fraud occur most often?".

Analyzes of secondary data from the questionnaire

Food scandals that happened in recent years have increased consumers' risk perceptions of foods and decreased their trust in food safety. A better understanding of consumer trust in food safety can improve the effectiveness of public policy and allows the development of the best practice in risk communication **(Kozelová et al., 2013a)**.

Among 354 respondents who participated in our questionnaire survey, 221 respondents (62.4%) replied to the first question concerning the commodity, that they believe to be the most adulterated is meat and meat products, which is partially in agreement with our results. Some respondents (26.02%) decided on milk and dairy products. Other commodities represented less than 10% (Figure 2).

The safety of milk has always been challenged due to the illegal use of preservatives and adulterants such as hydrogen peroxide, salicylic acid, benzoic acid, water, neutralizers, melamine, and so on **(Parminder and Gandhi, 2015)**.

At the question of which of the following causes of food fraud occur most often, respondents could select two options. 226 people (63.7%) decided for substitution, 142 people (40%) for mislabelling and 136 people (38.3%) for origin masking. Also, these results are partially in agreement with our results. Other types of adulteration received less than 100 votes (Figure 3). The question in which of these countries are food frauds reported mostly, they could also select two options. 310 people (87.6%) decided for Poland, 105 people (29.6%) voted for Slovakia and 43 people (12.1%) for Spain. Other countries received less than 10% (Figure 4). By the **RASFF-Annual report (2018)** there were 47 notifications on *Salmonella* in poultry products originating from Poland, most often (34 notifications) concerning *Salmonella* Enteritidis in fresh poultry. Two operators were identified as recurrent.

EU citizens have the right to be protected from fraudulent practices and to receive accurate information about the food they choose to purchase (**Vaqué and Vidreras, 2018).**

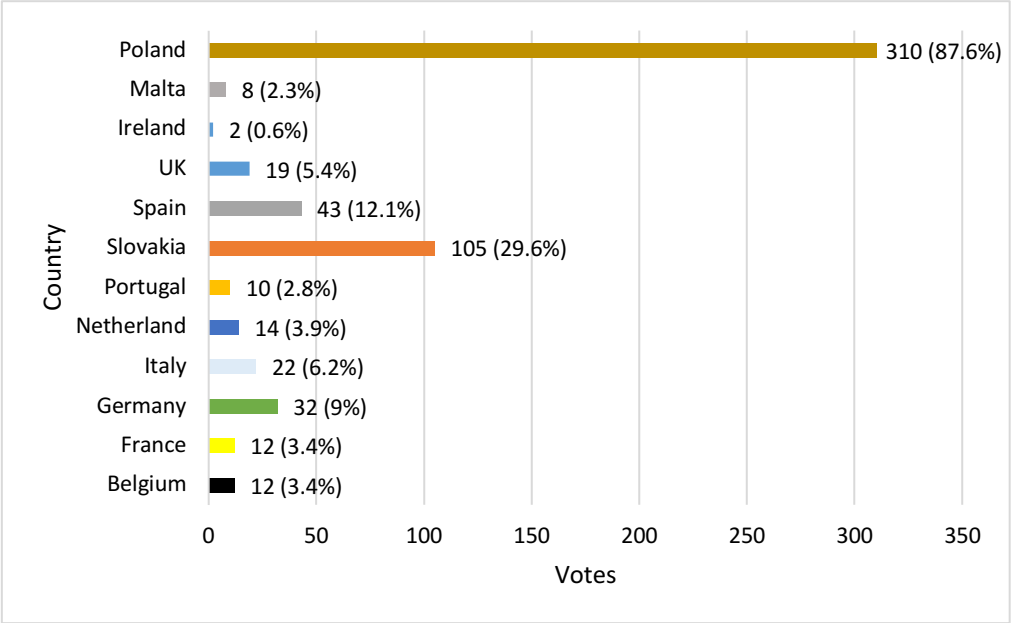

Figure 4 The answer to the question „in which of these countries are food frauds reported mostly?“

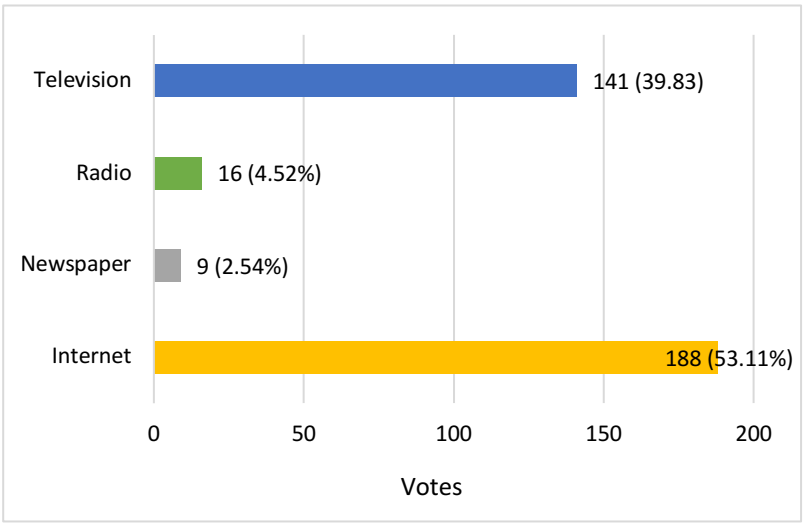

Figure 5 The answer to the question „from which media do you often receive information about food fraud“

So the last question was given about where your information is coming from 188 people (53.11%) select the Internet source and 141 (39.83%) the television source. Radio and newspaper as the source had the lowest number of votes (Figure 5).

CONCLUSION

The present work was focused on the current problems related to food fraud in the European Union countries. We have established hypotheses and confirmed their correctness. Of the total number of 163 cases during the period from 2017 to 2019 registered in KCFFQ, the most reported cases were in Italy and Spain. The most common commodities covered by these reports were fish and their products and meat and meat products. As the most common cause of food fraud was mislabelling. Based on confirmed hypotheses, we conclude that it is not statistically conclusive that the country has an influence on what type of fraud was performed but it has shown the link between the country and the food that has been adulterated. Also, there was no statistically confirmed correlation between countries and years when cases were reported, but a statistically significant correlation between years, commodities and the types of fraud that were reported. The results achieved in the evaluation of the responses from the individual questionnaires and the KCFFQ data indicate some level of information about food fraud topics. These results can be used as a basis for further investigation.

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THE EFFECT OF ANTIOXIDANTS ON THE QUALITY OF SEMI-FINISHED MINCED RABBIT MEAT

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ABSTRACT
Under the current adverse environmental conditions, enrichment of the human diet with essential nutrients, including antioxidants, which exhibit immunostimulatory and adaptogenic properties and protect the body against the negative effects of free radicals, is extremely relevant. Their use in food production, including that of semi-finished meat products, improves the quality of such products and extends their shelf life. It is of scientific and practical interest to enrich rabbit meat with the antioxidant-containing plant raw materials and to study their influence on the quality parameters of semi-finished products. In this work, modern, standard, commonly accepted methods of research were used to fulfill the stated objectives. Statistical processing of the results obtained and evaluation of the reliability of the data was carried out by statistical methods using IBM SPSS Statistics for Windows. This study demonstrates the expediency of using grape-seed powder, green tea extract, and amaranth/flax flour in semi-finished rabbit meat products. The optimum component composition and amount of multiple supplements to add to the semi-finished product were determined. The total phenolic and flavonoid content and antioxidant characteristics of the test samples were studied. The highest antioxidant potential was observed in samples with flax-containing multiple supplements. This paper demonstrates that microbiological indicators in all samples throughout the storage period, in line with hygienic requirements, were lower than those in semi-finished products containing multiple supplements as compared with a reference sample, while organoleptic indicators of quality were more stable. The content of toxic elements indicates the sanitary reliability of semi-finished products. Determination of the acid number and peroxide number values during storage revealed high resistance of semi-finished products containing multiple supplements to the accumulation of free fatty acids and peroxide compounds. The obtained data indicate the effectiveness of using developed semi-finished products as antioxidant products in the diet of the population.

Keywords: minced rabbit meat; plant raw materials; antioxidant properties; oxidation processes; quality indicators

INTRODUCTION
The maintenance of population health and quality of life is a major task of the world today. Scientific evidence has established that 10% of human health depends on the health system, 20% on hereditary factors, 20% on environmental factors, and 50% on human lifestyle and nutrition. Under the current adverse environmental conditions, enrichment of the human diet with essential nutrients, whose deficiency in the body leads to decreased immunity and metabolic disorders, as well as cardiovascular, oncological, and other diseases, is extremely relevant. In this regard, along with proteins, vitamins, and minerals, antioxidants, which have a wide range of biological effects, also deserve attention: they protect the body against the negative effects of free radicals and have immunostimulant, cardioprotective, anti-tumor and adaptogenic properties. **(Lapin et al., 2007; Gichev and Gichev, 2012; Packer, 2001; Plavinsky and Plavinskaia, 2013; Tarakhovsky et al., 2013)**. It is believed that despite their high effectiveness, some synthetic antioxidants have adverse effects on human health; however, antioxidants of natural origin, including polyphenolic compounds found in plant raw materials, are minimally toxic and do not cause adverse reactions **(Carocho and Ferreira, 2013; Kumar et al., 2013; de Oliveira et al., 2018; Shahidi and Ho, 2005; Shebis et al., 2013)**. The use of plant supplements that contain functionally different antioxidants is very important in the production of healthy foods, including semi-finished meat products. Preventing the accumulation of harmful oxidative products, they reduce losses resulting from oxidative spoilage and extend their shelf life **(Denisovich, 2006; Maqsood et al., 2013; Nasonova, 2008; Nikitina et al., 2011; Stratil et al., 2006; Tomović et al., 2017)**. In the food industry, antioxidant activities are the result of interactions between air and oxygen; they are also used to increase the microbiological stability of foods and improve their quality indicators **(Banerjee et al., 2017; Kuzmina et al., 2017; Mandro et al., 2009; Poliakov et al., 2017)**.

Among products containing natural antioxidants that can be used as an antioxidant supplement in semi-finished minced meat products, of particular importance are grape seed and green tea extract, which are characterized by a high polyphenol content **(Caleja et al., 2017; Higdon and Frei, 2003; Perumalla and Hettiarachchy, 2011; Shebis et al., 2013; Yashin et al., 2009)**. Together with natural antioxidants, we believe it is appropriate to use flax and amaranth as a functional supplement, as a source of plant protein enriching and additional biologically active ingredients **(Bernacchia et al., 2014; Kalač and Moudrý, 2000; Kidyaev et al., 2017; Oomah, 2001; Pastor and Acanski, 2018; Pękal and Pyrzynska, 2014; Perumalla et al., 2011)**. Therefore, it is of scientific and practical interest to enrich rabbit meat with the above-mentioned supplements and study their influence on the quality parameters of semi-finished products.

The work aims to develop technology to produce semi-finished rabbit meat products with antioxidant properties using a multiple plant supplement.

Scientific hypothesis

Scientific hypothesis

The addition of multiple supplements to semi-finished rabbit meat products will increase their resistance to the accumulation of oxidative products and improve quality indicators.

MATERIAL AND METHODOLOGY

The studies were carried out in the laboratories of the Department of Food of Akaki Tsereteli State University and the Department of chemical analysis and food safety of Shota Rustaveli State University. The study included natural semi-finished minced rabbit meat products made according to traditional recipes and model semi-finished and finished rabbit meat products containing plant supplements made according to recipes and technologies that we developed.

Test samples: 1 – Onion-containing semi-finished product; 2 – Fried onion-containing semi-finished product; 3 – Steamed onion-containing semi-finished product; 4 – Semi-finished product containing grape-seed and amaranth flour with multiple supplements; 5 – Fried semi-finished product containing grape-seed and amaranth flour with multiple supplements; 6 – A steamed semi-finished product containing grape-seed and amaranth flour with multiple supplements; 7– Semi-finished product containing grape-seed and flax flour with multiple supplements;

8 – Fried semi-finished product containing grape-seed and flax flour with aultiple supplements; 9 – A steamed semi-finished product containing grape-seed and flax flour with aultiple supplements.

Total phenolic compounds were defined using Folin-Ciocalteu spectral methods **(Stratil et al., 2006)**. The total flavonoid content (TFC) was determined by the aluminium chloride colorimetric method as previously described **(Pękal and Pyrzynska, 2014)**. Antioxidant activity was determined by using the DPPH (2,2-Diphenyl-1-pic-rylhydrazil) method **(Okawa et al., 2001)**.

During microbiological analysis, the quantities of mesophilic aerobic and facultative anaerobic microorganisms in rabbit meat were determined according to the state standard "Food products. Methods for determining the quantities of mesophilic aerobic and facultative anaerobic microorganisms" – State Standard **GOST 10444.15-94**. The number of intestinal bacilli was determined according to the state standard "Food products. Methods for the detection and determination of the number of bacteria of the *Escherichia coli* group (coliform bacteria)" – State Standard **GOST 30518-97/GOST P 50474-93**. *Salmonella* was determined according to state standard "Food products. Methods for the detection of bacteria of the genus Salmonella" – State Standard **GOST 30519-97**. The heavy metal content was determined on a SHIMADZU AA-6200 atomic adsorption spectrophotometer, the lead content by State Standard **GOST 26932-86**, the cadmium content by State Standard **GOST 26933-86**, the mercury content by State Standard **GOST 26927-86** and the arsenic content by State Standard **GOST 26930-86**.

The peroxide number value was determined according to State Standard **GOST 8285-91**. The method is based on the interaction of oxidative products of animal fats (peroxides and hydro-peroxides) with iodic potassium in a solution of acetic acid and isooctane or chloroform, with subsequent quantitative determination of iodine released by a solution of sodium thiosulfate using the titrimetric method.

The fatty acid value was determined according to the standard **GOST 13496.18-85**. The method is based on the neutralization of free fatty acids extracted from the product by a mixture of chloroform and ethyl alcohol with a 0.1 $mol.L^{-1}$ solution of potassium hydroxide.

Organoleptic indices were determined on a scale of 1 to 9 according to the following characteristics: appearance, colour, smell, taste, consistency, and juiciness.

Statistical analysis

To analyse the test parameters (total phenolic and total flavonoid content in the test samples, a correlation between total phenols and antioxidant activity) of natural semi-finished minced rabbit meat products, a statistical analysis was conducted of the obtained data, and the reliability of the obtained data was evaluated by statistical methods using the Windows IBM SPSS Statistics software program (version 20.0). To describe the continuous variables, we used statistical functions of the mean and standard deviation. Graphical interpretation of the results was carried out by using Microsoft Excel. The results of statistical analysis are presented in Tables 1 – 5 and Figures 1 – 4, and each value is an average of at least 10 determinations. Then, we computed the error of each measurement and calculated the squared errors to compute the absolute measurement error. We selected the value of reliability $p = 0.95$. Based on the number of measurements and the value of reliability, Student's coefficient equals $t = 3.77$ (Figure 2 and Figure 3) **(Romanov and Komarov, 2002)**.

RESULTS AND DISCUSSION

Pursuant to the stated goal, in the first stage of this work, we selected the main recipe components for the product. To prepare the reference samples, we added rabbit fat and spices to minced rabbit meat. The main ingredients in the reference sample recipes were minced rabbit meat, rabbit fat, onion, grape-seed powder, green tea extract, and amaranth/flax flour as multiple supplements. The multiple supplements were produced from grape-seed powder and hydrated amaranth/flax flour. Hydration was carried out in green tea extract - Hydromodulus 1:3 (1part of flour on 3 parts of extract). The multiple supplements were produced from grape-seed powder and hydrated amaranth/flax flour. Hydration was carried out in green tea extract - Hydromodulus 1:3 (1 part of flour on 3 parts of extract). The ratio of ingredients in multiple supplements was determined based on the optimum organoleptic parameters of semi-finished products under study.

The component and quantitative contents of multiple supplements are shown in Table 1. The amount of multiple supplements added to 100 g of semi-finished product was 30.2 g in the case of amaranth and 25.8 g in the case of flax flour.

In all samples except the one that did not contain a plant-based supplement, we determined the total phenolic content, flavonoid content, and antioxidant characteristics. To determine changes in these parameters as a result of heat treatment, meat was steamed. For this purpose, we took 9 test samples. The results obtained are illustrated in Figures 1 – 4. As can be seen from the diagrams, according to the total phenolic (Figure 1) and flavonoid (Figure 2) contents, samples with the flax supplements had the best characteristics. Since the contents of grape-seed and green tea extract in all test samples were almost identical, this difference can be explained by the chemical composition of flax, in particular, by the content of phenolic compounds and flavonoids.

Table 1 Components and quantitative composition of a multiple supplement.

No	Name of component	Content, %
1	Grape seed	0.7
2	Amaranth flour/flax flour	24.8
3	Green tea extract	74.5

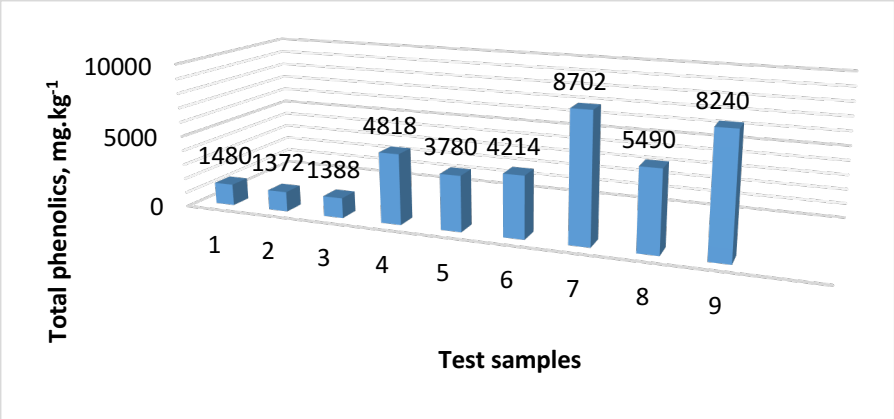

Figure 1 Total phenolic content in the test samples.

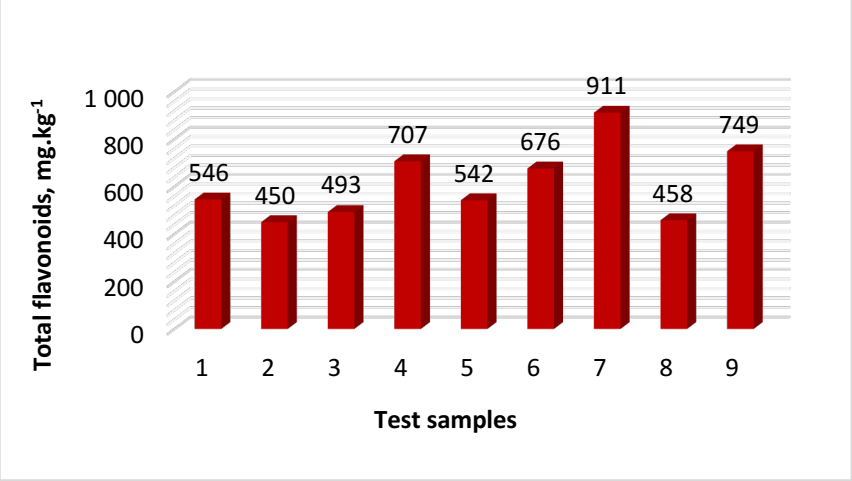

Figure 2 Total flavonoid content in the test samples.

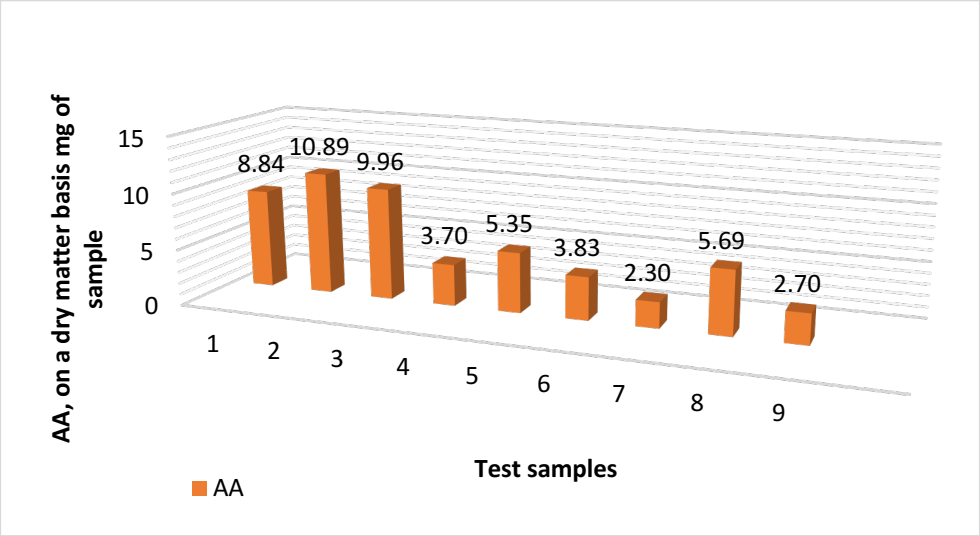

Figure 3 Antioxidant activity on a dry matter basis mg of sample.

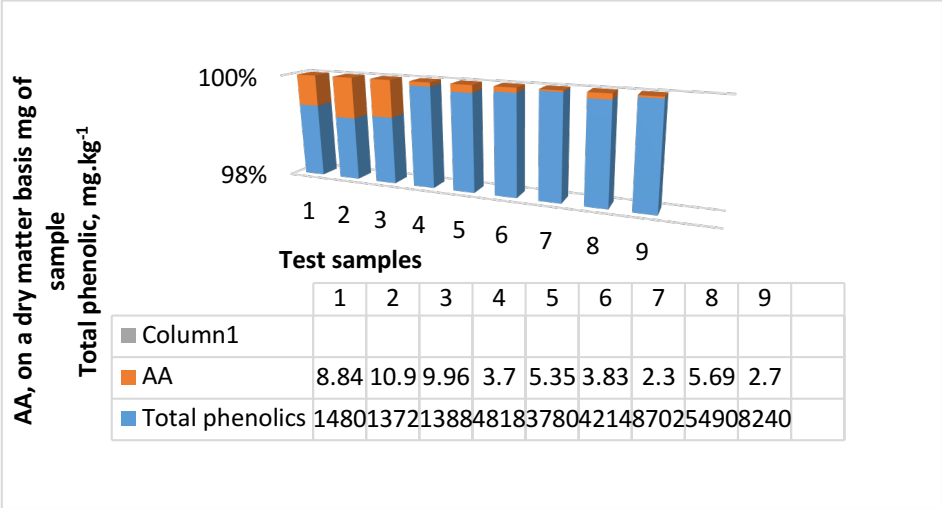

Figure 4 The correlation between total phenols and antioxidant aactivity.

The component and quantitative contents of multiple supplements are shown in Table 1. The amount of multiple supplements added to 100 g of semi-finished product was 30.2 g in the case of amaranth and 25.8 g in the case of flax flour. In all samples except the one that did not contain a plant-based supplement, we determined the total phenolic content, flavonoid content, and antioxidant characteristics. To determine changes in these parameters as a result of heat treatment, meat was steamed. For this purpose, we took 9 test samples. The results obtained are illustrated in Figures 1 – 4. As can be seen from the diagrams, according to the total phenolic (Figure 1) and flavonoid (Figure 2) contents, samples with the flax supplements had the best characteristics. Since the contents of grape-seed and green tea extract in all test samples were almost identical, this difference can be explained by the chemical composition of flax, in particular, by the content of phenolic compounds and flavonoids.

The total phenolic and flavonoid (Figure 2) contents were highest in semi-finished products (samples 1, 4 and 7), and after heat treatment, the value of phenolic compounds decreased by 5.3% in a steamed flax-containing sample (9) and by 36.9% in a fried flax-containing sample (8), and accordingly, the total flavonoid content decreased by 17.8% in the sample (9) and by 49.7% in the sample (8).

The phenolic compound content was 12.5% lower in the steamed amaranth-containing sample (6) and 21.5% lower in the fried sample (5). The total flavonoid content decreased by 4.4% and 23.3%, respectively. In the onion-containing samples, after their heat treatment, the phenolic compound content decreased by 7.3% in the fried sample (2) and by 6.2% in the steamed sample (3), while the flavonoid content decreased by 17.6% and 9.7%, respectively. The higher content of polyphenols in the steamed products was due to the use of the efficient mode of thermal treatment.

Figure 3 illustrates the antioxidant characteristics of a test sample. It shows that the highest antioxidant potential was observed in the sample containing the flax supplement. This would suggest that samples containing the flax supplement will be oxidized more slowly than other samples.

The diagram illustrating the correlation between total phenols and antioxidant activity (Figure 4) shows that the higher the total phenol phenolic content in samples, the higher their antioxidant activity (AA).

Table 2 Microbiological indicators of semi-finished products during storage.

Shelf-life, days	Mesophilic-aerobic and facultative anaerobic counts, cfu.g-1 (colony-forming unit per gram), less than			
	Control sample	Onion-containing semi-finished product	Amaranth-containing semi-finished product	Flax-containing semi-finished product
0	5.3×10^2	3.8×10^2	3.4×10^2	3.0×10^2
36	2.5×10^3	2.3×10^3	1.9×10^3	2.0×10^3
54	2.8×10^3	2.7×10^3	2.2×10^3	2.2×10^3
72	3.1×10^3	3.0×10^3	2.6×10^3	2.5×10^3
90	3.6×10^3	3.3×10^3	3.0×10^3	2.9×10^3

Table 3 Content of toxic substances in rabbit meat semi-finished products.

Indicators	Acceptable norm, $mg.kg^{-1}$	Semi-finished product		
		Onion-containing semi-finished product	Amaranth-containing semi-finished product	Flax-containing semi-finished product
Lead	Up to 0.5	0.08 ±0.01	0.05 ±0.01	0.07 ±0.003
Cadmium	Up to 0.05	<0.01	-	-
Arsenic	Up to 0.1	<0.0025	-	-
Mercury	Up to 0.03	0.011 ±0.005	0.015 ±0.001	0.01 ±0.005

Table 4 Change in the peroxide number values of semi-finished products during storage at a temperature of -18°C.

Shelf life, full day	Peroxide number value, mmole(1/2O_2)/kg			
	Reference sample	Onion-containing semi-finished product	Amaranth-containing semi-finished product	Flax-containing semi-finished product
0	0	0	0	0
12	0	0	0	0
24	1.45	0.65	0.58	0.52
36	1.76	0.83	0.72	0.67
48	2.68	1.56	1.03	0.96
60	4.5	1.75	1.67	1.43
72	5.28	2.12	1.93	1.74
90	7.34	2.96	2.32	2.15

Table 5 Change in the acid-number values of semi-finished products during storage at temperature of -18 °C.

Shelf life, days	Acid-number value, mg KOH/g			
	Reference sample	Onion-containing semi-finished product	Amaranth-containing semi-finished product	Flax-containing semi-finished product
0	0.47	0.39	0.18	0.21
12	0.83	0.76	0.33	0.31
24	0.98	0.87	0.52	0.37
36	1.36	1.15	0.54	0.46
48	1.43	1.21	0.57	0.51
60	1.59	1.42	0.65	0.62
72	1.78	1.59	0.74	0.71
90	2.89	2.38	1.22	1.11

Accordingly, the semi-finished rabbit meat products exhibit antioxidant properties due to the presence of a multiple supplement additive in the recipe.

When storing semi-finished meat products, it is essential to avoid bacteriological contamination. Thus, at the next stage of this work, we studied changes in microbiological indicators of minced semi-finished products of the developed rabbit meat under low temperature (-18 °C) storage conditions: the mesophilic-aerobic and facultative anaerobic count, colon bacillus group (Coliforms) bacteria, pathogenic microorganisms, including salmonellas (Table 2).

The data obtained indicate that throughout the storage period, the mesophilic-aerobic and facultative anaerobic count, in all samples, being in compliance with the hygienic requirements of microbiological safety, was less in semi-finished products containing multiple supplements than in the reference samples. This allowed us to conclude that the multiple supplements had an inhibitory effect on the development of microorganisms.

No colon *Bacillus* group bacteria or pathogenic microorganisms, including salmonellas, were found in samples, which also indicates the safety of semi-finished products.

Under today's difficult environmental conditions, food safety issues are of high relevance. Therefore, we identified the content of the toxic substances in the meat of rabbit breeds under study (Table 3). An analysis of Table 3 shows that the contents of lead, cadmium, arsenic, and mercury in different rabbit breeds did not exceed acceptable standards, which indicates their safety, as well as their sanitary and hygienic reliability.

It is known that lipid oxidation processes occur in meat products during storage, resulting in the accumulation of superoxide compounds. Therefore, we studied the dynamics of changes in the peroxide number reflecting the intensity of oxidative processes during storage at a low temperature (-18 °C). In parallel, we determined the acid-number value, which indicates the formation of free fatty acids in the products as a result of the hydrolytic spoilage of fats. As a reference sample, we took natural minced rabbit meat semi-finished products. The results are shown in Tables 4 and 5.

The analysis of these tables showed that the storage of rabbit meat semi-finished products was accompanied by an increase in acid-number and peroxide values. However, these processes were more intense in a reference sample than in the samples containing onions and multiple supplements. In particular, after 48 days of storage, the acid- number value was 15.4% lower than in a sample containing the onion supplement, 60.1% lower in a sample containing multiple supplements with amaranth, and 64.3% lower in the sample with flax-containing multiple supplements. After 90 days of storage, the acid- number values of samples with aultiple supplements containing onion, amaranth and flax flours were 17.6%, 57.8% and 61.6% lower, respectively, and remained within permitted limits.

The difference between the acid-number values in the amaranth- and flax-containing samples, in our opinion, was due to the fact that flax flour contains more phenolic compounds than amaranth **(Bernacchia et al., 2014; Kalač and Moudrý, 2000; Kidyaev et al., 2017; Oomah, 2001; Pastor and Acanski, 2018; Pękal and Pyrzynska, 2014)**.

The dynamics of change in peroxide number values during the storage of semifinished products were approximately similar. Particularly, after 48 days of storage, the mean peroxide number values as compared with reference samples were 41.8%, 61.6%, and 64.2% lower in samples with the onion, amaranth and flax supplements, while after 90 days of storage, these values were 59.7%, 68.4% and 70.7% lower, respectively.

The higher stability of the test samples as compared with the reference samples to the accumulation of free fatty acids and peroxide compounds is explained by the presence of plant supplements with antioxidant activity in their composition that slows the rate of these processes.

The results obtained confirm similar data available in the literature. For example, the addition of grape-seed powder, green tea extract **(Perumalla et al., 2011; Reshetnik et al., 2014)**, dihydroquercetin **(Kuzmina et al., 2017; Nasonova, 2008)**, pignoli nut extract nut **(Litvinova, 2012)** and rosemary **(Sharigina, 2011)** to semi-finished meat products prevents the accumulation of oxidative products and protects semi-finished products against oxidative spoilage. According to **Sharigina (2011)**, this is caused by the impact of the supplement's active components (phenolic diterpenes, including carnosic acid, essential oils, bioflavanoids, catechins) on chain reactions.

We studied organoleptic indicators of the quality of semi-finished and finished products. After 60 days of storage of the semi-finished products, the reference sample had a more pronounced foreign flavour as compared with samples containing multiple supplements, which perhaps was due to faster accumulation of oxidative products in the reference sample. As to colour stability, after 60 days of storage, it was more stable in samples containing multiple supplements, which was due to the presence of ingredients with antioxidant properties.

The finished product had better consistency and juiciness than the reference sample, which is explained by the high water-retention and water-binding ability of amaranth and flax flour **(Carocho, Morales and Ferreira, 2018; Denisovich, 2006; Higdon and Frei, 2003; Tarakhovsky et al., 2013; Tavdidishvili et al., 2018; Vaitanis and Khodyreva, 2017)**.

Based on the above, samples containing multiple supplements, both semi-finished and finished products had better organoleptic indicators of quality during storage.

Thus, a multiple plant supplement that protects products against microbiological and oxidative spoilage and improves their quality imparts the antioxidant properties to the developed semi-finished products, demonstrating the effectiveness of their use in the production of healthy semi-finished meat products.

CONCLUSION

1. To impart antioxidant properties to minced semi-finished rabbit meat products, we have proven the effectiveness of using grape-seed powder, green tea extract, and amaranth/flax flour as multiple supplements in the recipe.
2. We have determined the optimum component composition of multiple supplements to added to semi-finished rabbit meat products: 0.7% grape-seed powder, 24.8% amaranth flour/flax flour, and 74.5% green tea extract. The amount of multiple supplements to be added to 100 g of semi-finished product is 30.2 in the case of amaranth flour and 25.8 g in the case of flax flour.
3. In the test samples, we studied the total phenolic and flavonoid content. It has been established that the highest antioxidant potential was observed in samples with flax-containing multiple supplements.
4. Microbiological indicators of semi-finished products with multiple supplements throughout storage at low temperature were in line with the hygienic safety requirements, and organoleptic indicators of quality were more stable as compared with a reference sample. The content of toxic elements indicates the sanitary reliability of semi-finished products.
5. The higher stability of semi-finished rabbit meat products with a control sample to the accumulation of free fatty acids and peroxide compounds has been established.

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THE USE OF BIOFORTIFICATION FOR PRODUCTION OF SELENIUM ENRICHED GARDEN PEA

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ABSTRACT

Biofortification of crops with selenium is one of the possible manners on how to increase selenium intake by humans. The effect of selenium fertilization in relation to selenium enrichment of pea and following the phytotoxicity symptoms in garden pea plants was studied. Pot experiments were established with a control variant without selenium addition and four variants where selenium was applied as sodium selenate into the soil in four different concentrations (1 – 6 mg Se.kg^{-1}) before seeding. Garden pea was grown in pots for 60 days and then plant material was dried and submitted to analysis. The total content of selenium was determined by the ZET-AAS method in the roots, above-ground parts of the plant (stems, leaves, extracted pods), and in seeds of a pea. Dean-Dixon´s test and paired t-test (α = 0.05) were used for statistical evaluation of the results. Transfer factors were calculated as a ratio between selenium content (mg.kg^{-1}) in individual plant material and soil. Transfer indexes were calculated as a ratio between selenium content (mg.kg^{-1}) in seeds and roots. The results showed that with the increasing addition of the Se to the soil, its contents in all parts of the plant proportionally increased. The content of the Se increased in the roots 43 to 173-fold, in the above-ground parts 79 to 372-fold, and in the seeds Se was accumulated 130 to 415 times more compared to control. Transfer factors and transport indexes were expressed. Transfer factors for pea varied from 11.05 to 19.25 in the case of Se transfer to the whole pea biomass. In the case of the Se transfer from soil to pea seeds, the highest transfer showed variant with addition 1 mg Se.kg^{-1} and the transfer factor gradually decreased with increasing addition of Se. Based on the amount of biomass produced, the experiments statistically confirmed the phytotoxicity of higher doses (4 and 6 mg Se.kg^{-1}) of selenium to plants. The highest transport index values are shown variants with the Se addition 1 and 2 mg Se.kg^{-1} (2.03 and 1.77, respectively). In these variants, Se was used the most efficiently. Our results showed that the best biofortification results were obtained in experimental variants with the lower selenium additions (1 and 2 mg Se.kg^{-1}).

Keywords: biofortification; selenium; garden pea

INTRODUCTION

Selenium (Se) is an essential micronutrient, which is involved in selenoaminoacids and selenoproteins. It has been proved to have multiple roles in the growth and functioning of living cells and has many crucial biological functions in animals and humans **(Birringer, Pilawa and Flohé, 2002; Tapiero, Townsend and Tew, 2003)**. As a cofactor of the enzyme glutathione peroxidase, and a catalyser of the reduction of peroxides, plays Se role in antioxidant defence. However Se is biologically active at low concentrations for normal growth and development, and at moderate concentrations for homeostatic function, at high concentrations, Se can cause toxicity **(Puccinelli, Malorgio and Pezzarossa, 2017)**. The proposed Recommended Dietary Allowance (RDA) of 55 µg Se day^{-1} for adults and a tolerable upper intake of 400 µg Se day^{-1} has been set by The Food and Nutrition Board of the Institute of Medicine (USA) **(Krinsky et al., 2000)**.

Central Europe has been identified as a region with suboptimal concentrations of selenium in agricultural soils that reflects in low selenium levels in the agricultural products coming from the area **(Sager, 2006)**.

In the past, great efforts have been focused only on increasing crop yields, but enhancing the concentrations of mineral micronutrients including selenium has become an urgent task. Biofortification of trace elements can be achieved by their application within the agronomic process such as soil or foliar fertilization or crop breeding **(El-Ramady et al., 2014)**. Agronomic biofortification with Se could be a powerful tool to remedy Se deficiency as it may increase the Se concentration in the plant sources use in food production **(Poblaciones, Rodrigo and Santamaría et al., 2015)**. Use of common fertilizers with selenium (Se) for crop production is considered as an effective way to produce selenium-rich food and feed.

Legumes and cereals present the basis of the diet of billions of people. Legumes are able to accumulate

micronutrients that create the potential to be used in Se biofortification programs **(Thavarajah, Ruszkowski and Vandenberg, 2008; Thavarajah, Warkentin and Vandenberg, 2010)**. **Poblaciones, Rodrigo and Santamaria (2013)** confirmed that the pea has a strong ability to uptake and accumulate Se; therefore, it could be considered as a very strong candidate for inclusion in biofortification programs aiming to increase Se in the food chain. The value of green pea seeds and forages as an alternative protein source can be improved by using agronomic biofortification **(Garousi et al., 2017)**. Biological changes of garden pea (*Pisum sativum* L.) in dependence with the application of inorganic form of Se (and sodium selenate) at different concentrations have been studied. As a plant material was garden pea (*Pisum sativum* L.) that has been recognized as an important source of vitamins C, E, and a group of B vitamins including folic acid derivatives, so-called folates and mineral substances (Fe, K, P, Mn, Mg, Ca). Peas is also rich for soluble and insoluble fiber, vegetable protein and is a source of the macro elements **(Chrenková et al., 2003)**. It has highly valuable in proteins that are similar to those of animal origin. Also, the biological value of the pea protein is higher than that of the cereals. Both pea seeds and forages are rich in protein including lysine and other essential amino acids **(Garousi et al., 2017)**.

Our purpose was to investigate the most efficient selenium additions into the soil to achieve enhancement of Se levels in pea plants as well as identification of potential phytotoxic effects on the plants caused by different levels of inorganic selenium applied.

Scientific hypothesis

Increasing selenium additions in soil reflect in increasing selenium content in pea plants. Higher selenium additions have a potential phytotoxic effect on pea plants.

MATERIAL AND METHODOLOGY

Pot experiments with garden pea were carried out on soil biofortification by sodium selenate. As an experimental material was selected the garden pea (*Pisum sativum* L.) – the Oskar variety (Semo, The Czech Republic). This variety matures relatively early and is suitable for direct consumption and industrial processing, as well. In Slovakia, it is the variety produced intensively in agricultural conditions.

Pot experiments in the phytochamber were based on five variants and eight repetitions. Sodium selenate was applied to the soil substrate 5 days before the planting of pea seeds, in quantities 0, 1, 2, 4, and 6 mg $Se.kg^{-1}$ soil. On the fifth day, we planted 25 seeds of the pea in each container and placed them in the phytochamber with controlled temperature and light regimen. In the stage of technological maturity (60 days after the inception of the experiment) were the plants harvested and the plant material was separated into the total above-ground part, seeds and roots. A soil sample from each pot was taken for analysis and the separated plant material was weighed for each variant for the conversion of the Se content to the total biomass of peas.

The content of the Se has been determined in the roots, above-ground part of the plant (stems, leaves, extracted pods) and grain of peas. Dried soil and plant samples were wet decomposed in the mixture of nitric acid and hydrogen peroxide and after dilution measured according to the previously optimized method described by **Hegedűs et al. (2008)**. The determination of the total selenium was done by atomic absorption spectrometry with Zeeman background correction (ZET-AAS). The apparatus used for analysis was AA240Z Varian (Mulgrave Virginia, Australia), selenium hollow cathode lamp was used for the measurement, and the wavelength was set at 196 nm.

Statistical analysis

Dean-Dixon´s test and paired *t*-test ($p < 0.05$, n = 8) were used for statistical evaluation of the results by a Tanagra 2.0 software.

RESULTS AND DISCUSSION

Pot experiments in the phytochamber were realized to monitor the transfer of Se to the pea plant in model conditions. The results showed that with increasing addition of the Se to the soil, its contents in all parts of the plant proportionally increased (Table 1). Variants with an addition of 1 mg $Se.kg^{-1}$ and 2 mg $Se.kg^{-1}$, showed that the content of the Se was highest in grain, variants with 4 mg $Se.kg^{-1}$ and 6 mg Se.kg kg^{-1} had the highest content of the Se at the root. This fact can be probably linked to the slowdown of plant development due to the phytotoxicity of Se.

Further noted differences between the growth of the individual variants were in the total weight of the plant material where variants with 4 mg $Se.kg^{-1}$ and 6 mg $Se.kg^{-1}$ quantity of plant material was compared to the control 4.5 and 5.7 times lower. Also, the plants of the variants mentioned had at the time of harvesting the less developed seeds compared to the control and variants with the addition of 1 mg $Se.kg^{-1}$ and 2 mg $Se.kg^{-1}$. The content of the Se increased in all parts of the pea plant in variants I to IV, namely at the root of 43 to 173-fold, in the above-ground parts of 79 to 372-fold and in the grain Se was accumulated 130 to 415 times more compared to control. The analysis of the pea showed that selenium fertilization of soil with different selenium additions in the sodium selenate form resulted in a proportional increase of the total selenium content in all parts of the pea.

The statistical evaluation compared the means of the Se content within the different variants in the roots and in the grains. A statistically significant difference ($p < 0.05$) was not found only in the roots between variant I and II and between variants III and IV. For Variant IV, the roots showed symptoms of phytotoxicity and the roots were not able to uptake higher quantities of Se. The statistical evaluation of the Se content in a grain showed a significant difference ($p < 0.05$) between all the variants. The positive correlation between the transfer of Se content from the Se-fortified soil to the seeds of the garden pea reflects the graph of polynomic dependency in Figure 1.

We have found a tight positive correlation which is confirmed with a high coefficient of correlation (R = 0.9969) which was calculated from the coefficient of determination (R^2 = 0.9939) from the dependence of Se transfer from soil enriched with sodium selenate to garden pea seeds. With regard to doses applied, there was found

also a highly tight relationship between the Se content in other plant parts and the Se applied.

The positive effect of sodium selenate fertilization on enhancement of selenium content in peas confirmed also authors **Poblaciones, Rodrigo and Santamaria (2013), Poblaciones, Rodrigo and Santamaria (2015), Garousi et al. (2017), Hegedűsová et al. (2015), Smrkolj et al (2006)** and **Hegedűs, Hegedűsová and Šimková (2007)**.

Our experiments in phytochamber pointed at several times the higher accumulation of selenium in the conditions of regulated light regime compared to the experiments in greenhouse or field conditions. **Ros et al. (2016)** explored available results from selenium fertilization experiments in different conditions and across all observations, the strongest accumulation was achieved in the experiments in growth chambers, mainly in solutions. Probably this positive effect could be explained by even larger volatilization and transfer between the high Se-treatments to control and low Se-treatments during the growth.

Pot experiments with peas were realized with the same variants of the Se addition in outdoor conditions with normal light conditions, so the impact of UV-B should also be taken into account from the phytotoxicity point of view when comparing the results of experiments in the phytochamber and in the outer environment. UV-B radiation in normal light conditions for the accumulation of Se and the signs of phytotoxicity of the Se on the pea.

The Se phytotoxicity symptoms include the amount of biomass produced. The experiments carried out confirmed statistically the phytotoxicity of higher selenium doses (4 and 6 mg $Se.kg^{-1}$) to plants, based on the weight of the biomass produced is given in Table 2.

From the evaluation of the biomass weights, it can be assumed that the first signs of phytotoxicity begin to emerge after the application of 2 mg $Se.kg^{-1}$. The symptom was later maturation of the seeds compared to the control or variant I. The significant braking effect on the formation of biomass and seeds had Se additions 4 and 6 mg $Se.kg^{-1}$. The limit concentration with the phytotoxic effect on garden pea will probably be a selenium addition of 3 $mg.kg^{-1}$, which also confirmed the results of the work of **Vargová et al. (2009).**

In Figure 2, the phytotoxic effect of the Se addition in variants III and IV (4 and 6 mg $Se.kg^{-1}$) can be clearly observed. It was visible from the first days of plant germination and persisted throughout growing and ripening until the plants were harvested.

Table 1 Selenium content in pea and soil substrate - results of sodium selenate fertilization in the phytochamber.

Variant	**Plant part**	**Dry matter**	**Se content in dry matter**	**Total Se content in the whole phytomass**	**Se content in soil after experiment**
Se ($mg.kg^{-1}$ substrate)		(%)	mean ±*SD* ($mg.kg^{-1}$)	(mg/pot)	mean ±*SD* ($mg.kg^{-1}$)
K 0 mg Se	root	11.82	1.90 ±0.37	0.007	0.26 ±0.06
	above-ground part	19.55	0.56 ±0.24		
	seed	22.29	0.74 ±1.96		
I 1 mg Se	root	16.40	81.54 ±35.44*	1.204	1.32 ±0.47
	above-ground part	32.64	65.56 ±20.58*		
	seed	27.96	96.50 ±39.72*		
II 2 mg Se	root	14.57	121.34 ±45.79*	1.171	2.12 ±1.03
	above-ground part	21.21	108.32 ±7.99*		
	seed	19.92	137.68 ±21.74*		
III 4 mg Se	root	15.27	303.16 ±94.79*	1.633	3.18 ±0.48
	above-ground part	24.31	245.70 ±66.58*		
	seed	20.02	242.20 ±31.86*		
IV 6 mg Se	root	34.85	327.66 ±43.26*	1.587	4.84 ±1.34
	above-ground part	22.13	308.80 ±74.01*		
	seed	18.05	309.00 ±65.32*		

Note: *statistically significant difference (comparison with the control variant; $p < 0.05$, $t_{krit} = 2.365$).

Table 2 Total weight of the biomass produced for garden pea.

Plant part	**Weight of fresh biomass** (g/pot)				
	K	**I**	**II**	**III**	**IV**
	mean ±*SD*	**mean ±*SD***	**mean ±*SD***	**mean ±*SD***	**mean ±*SD***
root	17.18 ±2.28	19.85 ±3.59	18.19 ±4.59	7.41 ±2.33*	8.46 ±1.58*
above-ground part	36.14 ±5.62	37.75 ±7.13	32.88 ±3.83	20.27 ±1.77*	9.99 ±5.03*
seed	5.93 ±2.07	4.95 ±2.02	3.45 ±1.30*	1.48 ±0.26*	0.17±0.06*

Note: *statistically significant difference (comparison with the control variant; $p < 0.05$, $t_{krit} = 2.365$). Experimental variants: K = 0; I = 1; II = 2; III = 4; IV = 6 mg $Se.kg^{-1}$ substrate.

The selenium supplement in these variants caused by braking growth, later development of flowers, and delayed seed formation.

The flowering in variant III occurred approximately 4 days and in variant IV by 7 days later compared to the control variant K. The results indicated that the phytotoxicity of the salt applied was not observed in the germination but later at the stage of growth, probably when the young plant starts to behave autotrophic and received dissolved ions of selenate from the soil solution. The Se concentration at which the phytotoxicity begins to occur is different for each species of cultural plant. Determination of the concentration limit of Se phytotoxicity is dependent on the soil enrichment technology. In recent years, increased attention has been paid to the protective role of Se in the plant against the stress caused by the higher intensity of UV radiation. **Hartikainen and Xue (1999), Germ, Kreft and Osvald (2005)** and **Smrkolj et al. (2005)** described that appropriate levels of Se in plants help to protect against oxidative stress caused by increasing UV-B radiation. The results confirm that in plants under normal light conditions, the appropriate addition of selenium helps to produce a larger crop.

The activity of the antioxidant enzymes superoxide dismutase, guaiacol peroxidase, and glutathione reductase in cucumber leaves was studied by **Jain, Kataria and Guruprasad (2004).** They found that the activity of antioxidant enzymes increased in cucumber leaves as a

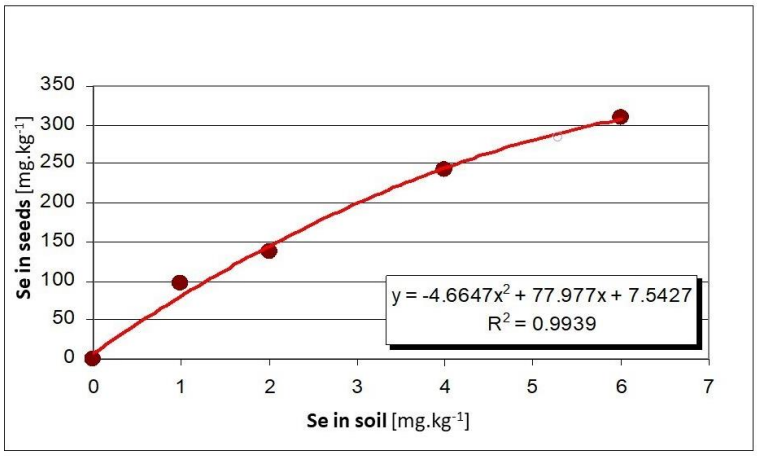

Figure 1 Dependence of Se transfer from soil enriched with sodium selenate to garden pea seeds.

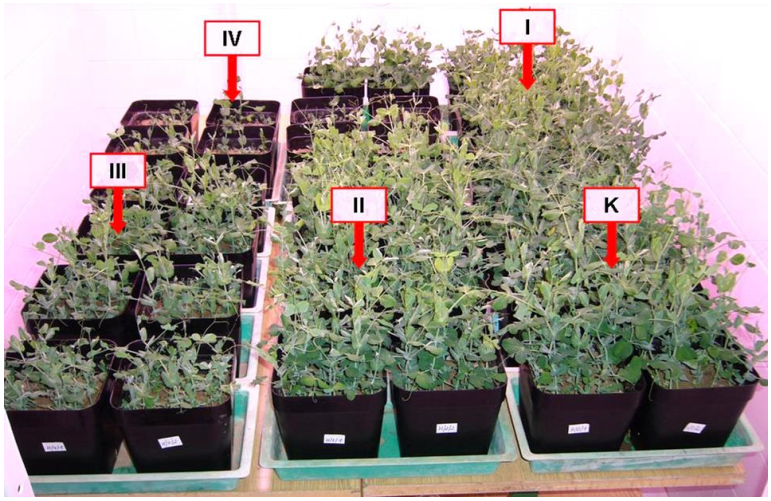

Figure 2 Visual comparison of individual experimental variants in phytochamber 20 days after experiment. establishment

Table 3 Selenium transfer factors and transport indices in garden pea.

Variant	Se addition ($mg.kg^{-1}$)	Mean content of Se in fresh biomass ($mg.kg^{-1}$)	TF_{SP}	TF_{SG}	TI_{RG}
K	0	0.17	0.00	0.00	0.79
I	1	19.25	19.25	25.03	2.03
II	2	22.09	11.05	13.76	1.77
III	4	57.20	14.30	12.19	0.98
IV	6	83.53	13.92	9.30	0.50

Note: TF_{SP} – transfer factor soil – plant, TF_{SG} – transfer factor soil – seeds, TI_{RG} – transport index root – seed.

response to the increasing intensity of UV-B radiation. Free radicals generated by UV-B radiation are likely to be associated with the induction of a defensive antioxidant system. The activity of UV-B induced enzymes is altered by the external application of antioxidants, which are also capable of scavenging free radicals and relieving plant stress due to UV-B radiation.

These facts lead to an explanation of the differences observed between the light regime during growth in the phytochamber (lower intensity of UV radiation) and normal outdoor light conditions. Increased cumulation of Se in peas in all variants compared to those grown under normal light conditions is evidence of this.

Transfer factor and transport index

The transfer factor (TF) is considered as an indicator of the element's entry into the plant. The concentration of chemical elements in soil and dry matter of plant samples are used for calculation **(Uchida, Tagami and Hirai, 2007; Blanco Rodríguez et al., 2006; Bitterli, Banuelos and Schulin, 2010)**. Transfer factors for peas varied from 11.05 to 19.25 in the case of Se transfer to the whole pea biomass (TF_{SP}), in the case of the Se transfer from soil to pea seeds (TF_{SG}), the highest transfer showed in variant I and gradually decreased with increasing addition of Se (Table 3). In general, transfer factors are higher than those reported in the literature. Literary data describe transfer factors under natural conditions without soil fortification, therefore selenium content in vegetables was naturally lower.

The transfer factor (TF) depends on the type of plant, part of the plant, and the soil characteristics as well as the chemical forms of the element in the soil **(Bitterli, Banuelos and Schulin, 2010)**. The leaves of plants have a higher transpiration rate, therefore they require more water and essential elements than other parts of the plant, and therefore trace elements are used to accumulate to a higher extent in the leaves. Therefore, there is a higher transfer factor in these parts of the plant compared to the others, as confirmed by several authors, **Uchida Tagami and Hirai (2007)** and **Blanco Rodriguéz et al. (2006)**.

Uchida Tagami and Hirai, (2007) reported TF in for 68 vegetable and cereal samples. At a mean Se content in the soil of 0.54 $mg.kg^{-1}$, cabbage had a TF of 0.011 – 0.036, soybean 0.016 and lettuce 0.017 – 0.0083.

A transport index (TI) is used to evaluate the ability of a plant to transport an element to a collectible portion of a plant. **Blanco Rodriguéz et al. (2006)** defines the transport index as the ratio of element concentration in above-ground resp. in the consumption part and its concentration in the root. The transport index was used to quantify the relationship of Se transportation from roots to grains. The Se concentration ratio in the grain to the concentration in the root in peas is expressed by the TI_{RG} values in Table 3. The highest TI_{RG} values are shown for variants I (2.03) and II (1.77). These variants used Se the most efficiently.

CONCLUSION

Our findings proved that Se biofortification has positive effects on the improvement of selenium status in garden pea that can provide Se enriched food source for the production of a proper functional product. The results suggest that peas are suitable vegetable species for agronomic biofortification and can introduce Se into the human and animal diet. Garden pea has a great ability to accumulate high amount of Se in the grain. Our investigations proved that a relatively small amount of Se fertilizer can highly enhance Se supply in the grain. A strong linear relationship between the total Se content in pea and the dose rates of the application were also proved, however from the phytotoxic point of view, concentrations over 2 mg Se kg^{-1} of soil resulted in a decrease of plant growth and delay of seed development. This fact presents valuable information for potential recommendations for agronomic practice. In conclusion, our results could be used as valuable information for suitability of peas for inclusion in Se biofortification programs; however, another additional research on the sustainability of biofortification with even low selenate addition into different soils should be considered in relation with a potential environmental impact on arable soils and surface waters, as well.

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RESEARCH OF MILK FAT OXIDATION PROCESSES DURING STORAGE OF BUTTER PASTES

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ABSTRACT

Basic quality indicators studied: acidity, peroxide, anisidine value and integrated value of complete fat oxidation. Butter paste was selected as a reference, consisting of butter, skim milk powder and fat-soluble emulsifiers. Peroxide value during storage at the temperature of (4 ±2 °C) for the first 4 days did not exceed 5.0 1/2 O mmol.kg^{-1}, on the 15th day fat peroxide value of butter paste with milk-vegetable protein exceeded permissible limits that is indicative of milk fat contamination. At the temperature of (-3 ±1 °C) butter paste fat couldn't be qualified as fresh when storing during 15 days, peroxide value exceeds permissible limits on the 25th day of storage. Rising of the peroxide value above 5 1/2 O mmol.kg^{-1} was detected on the 25th day of storage, exceeding of threshold value was on the 45th day. It was established that rate of oxidation processes in butter pastes with vegetable protein is the highest among all studied samples in each particular control and observation point. It was determined that the rate of secondary lipid oxidation depends on the storage temperature and is observed when storing butter paste samples at a temperature of (-3 ±1 °C) on the 10th day, (-24 ±2 °C) – on the 30th day of storage. Acid value did not exceed recommended limits (2.5 °K) and was on average – 2.3 °K when storing butter paste during 10 days at a temperature of (4 ±2 °C); 2.1 °K during 20 days at the temperature of (-3 ±1 °C), 2.4 °K during 40 days at the temperature of (-24 ±2 °C). In view of obtained results of fat phase stability evaluation of studied butter pastes, the following storage maximum time is recommended: at the temperature of (4 ±2 °C) – 7 days, at the temperature of (-3 ±1 °C) – 15 days, at the temperature of (-26 ±2 °C) – 30 days.

Keywords: butter paste; milk protein; vegetable protein; milk; fat; oxidation

INTRODUCTION

Relatively high calorific value of butter often serves as limitation when adding to food ration of a modern human and compels scientists and producers of dairy products to develop technologies similar to butter with lower fat content. Fat exclusion from the ration for a long term is inadvisable and dangerous in as much as lipids have exceptional value in human physiology. Lipids are source of energy and fulfil a range of functions in the body: heat insulation, protection of internal injury, exercise anatomic and tectonic function as part of cell membrane, arranging order of metabolites flow in the body **(Kubicová, Predanocyová and Kádeková, 2019)**. Biological value of fats is determined first of all by availability of polyunsaturated fatty acids – linoleic, linolenoic and arachidonic acids. These fatty acids are not synthesized by human body, if they are insufficient in food products lipid exchange processes fail in the body. Linoleic and linolenoic acids are contained primarily in vegetable fats, arachidonic – in animal fats **(Zahorui, Mazur and Kalinina, 2019; Bozhko et al., 2017)**. Milk fat is unique as it contains all three aforesaid fatty acids at once. Besides, fat-soluble vitamins, in particular vitamin A and β-carotene are also assigned to milk lipids. Therefore, development of new butter paste types which are low-caloric substitute of butter is topical trend of researches.

Since butter pastes are defined by high moisture contents, skim milk powder, milk or vegetable proteins, protein polysaccharide complexes etc. are used for their structure stabilization **(Kochubei-Lytvynenko et al., 2018a; Ondrušíková et al., 2019; Kumbár et al., 2017)**. Free moisture is suitable environment for biochemical transformations, that is why during production and storage of butter pastes there is a danger of biochemical transformations of milk fat and other components, primarily proteins and carbohydrates limiting storage life of such products **(Golian et al., 2018; Frolova et al., 2019)**.

One of the main processes occurring during storage of butter pastes and causing product spoilage is milk fat oxidation. Fatty acid content of milk fat exercises significant influence on preservation of butter pastes

during storage **(Dyman and Zahoruy, 2008; Bubelová et al., 2017)**. First of all polyunsaturated fatty acids are prone to oxidation under influence of atmospheric oxygen, causing accumulation of peroxide and carbonyl compounds. Therefore, the course of oxidation changes in developed butter pastes were studied when controlling peroxide and anisidine values. Oxidation stability of fat as part of a product also depends on production conditions, heat and light impact, concentration and type of oxygen, presence of free fatty acids, mono- and diglycerides, metals of variable valency, peroxides, thermal oxidized compounds, pigments and antioxidants. These factors mutually influence on the oxidation process and it is practically impossible to determine their individual effect, however at initial oxidation stages chemical and organoleptic indicators of fat have little or no change.

Fat oxidation process is free radical chain reaction, comprising initiation, branching and termination stage and which takes place involving molecular oxygen **(Piven, 2007)**:

Chain initiation	$H \rightarrow R\cdot + H\cdot$
Chain branching	$R\cdot + 3O_2 \rightarrow ROO\cdot$
	$ROO\cdot + RH \rightarrow ROOH + R\cdot$
Chain termination	$ROO\cdot + R\cdot \rightarrow ROOR$
	$R\cdot + R\cdot \rightarrow RR$
	(R – lipid radical)

Depending on storage conditions and initial qualities of product the oxidation may take place under different mechanisms. Products of described reactions are first and foremost hydroperoxides, from which aldehydes, ketones, short-chain hydrocarbons, spirits and ethers are formed by autoxidation mechanism. Hydroperoxides formed in the reaction with oxygen can be determined by peroxide value, products of their further transformation – by anisidine value **(Tsisaryk, Musiy and Shereshkov, 2016)**.

Lipids oxidation is followed by deep breakdown of triacyl glycerides with formation of peroxide compounds, aldehydes, ketones etc. At this time foreign flavours and odours appear in the product. Oxidation may be nonenzymic and enzymic. Nonenzymic oxidation is followed by peroxides accumulation at the beginning, thereafter aldehydes, ketones, oxyacids and other compounds, which add foreign flavours and odours to dairy products. This process takes place as a result of fat interaction with molecular oxygen. At first free milk butter not protected by membrane is exposed to oxidation, out of fatty acids – unsaturated ones. Oxidation process is controlled by peroxide value **(Javůrková et al., 2016; Korablova, 2011)**.

This value is very sensitive and is a reference for beginning and rate of fat oxidation. Fresh fat is free from peroxides. At initial oxidation stages for some time chemical and organoleptic indicators of fat are almost unchanged. During the storage fat begins spoiling. It can be detected by increase in peroxide value and change of organoleptic properties of fat **(Decker, 2002; Li et al., 2018; Saláková et al., 2019)**. End products of milk fat splitting are free fatty acids **(Martin-Polvillo, Marquez-Rui and Dobarganes, 2004)**, accumulation of which result in bad taste and odour of the product and acidity level increase. In addition to, accumulation of organic acids may be a result of deep breakdown of proteins and carbohydrate components, in particular lactose.

Thus, the study of the milk fat oxidation processes of butter pastes during storage at different temperatures will allow specifying conditions and storage life of products and ensuring product safety for a consumer.

Scientific hypothesis

Fat splitting process in butter pastes, stabilized by protein polysaccharide complexes, has the same tendency, but slightly slower as compared to butter paste, which contains skim milk powder. Process deceleration can be explained by reduction of free moisture content through using more active structure-forming agents and by synergism of their interaction.

MATERIAL AND METHODOLOGY

Material

Authors developed formulations of butter pastes, stabilized by protein polysaccharide complex and justified technological modes of their production. **(Kochubei-Lytvynenko et al., 2018a; Kochubei-Lytvynenko et. al., 2018b)**. Figure 1 represents the photo of a butter paste stabilized with protein-polysaccharide complex.

Figure 1 Butter paste stabilized with protein-polysaccharide mixture.

In order to ensure stable properties of butter pastes and prevention of their spoilage during storage, changes in quality during storage by acidity, peroxide (PV) and anisidine (AV) values were studied. Product samples were stored in an unsealed package at three technological modes: at a temperature of (4 ±2 °C) during 15 days, (-3 ±1 °C) during 25 days and at a temperature of (-26 ±2 °C) during 45 days at relative air humidity of not less than 85% without sunlight. Acidity was determined directly in collected sample, oxidation indicators – at fat extraction by digestion method after each 5 days of storage (3 days at a temperature of (4 ±2 °C). Butter paste was selected as a reference, consisting of butter, skim milk powder and fat-soluble emulsifiers.

Methods

Determination of fat peroxide value was conducted under State Standard of Ukraine DSTU 4570:2006 **(DSTU 4570, 2006)**. The method is based on the reaction of interaction of fat oxidation products (peroxides and hydroperoxides) with potassium iodide in acetic acid and chloroform solution and the subsequent quantitative determination of released iodine with sodium thiosulfate solution by the titrimetric method. For determination of peroxide number

such chemical reagent were used: distilled water, glacial acetic acid, chemically-pure (C.P.), chloroform (trichloromethane), aqueous solution of potassium iodide C.P. mass content 50 – 55% freshly prepared, solution of starch mass content 0.5%, aqueous solution of ($Na_2S_2O_3$. $5H_2O$) molarity 0.01 mol.L^{-1}, final of sodium sulphite with mass of substance in vacuum-sealed ampule , which equals 0.1 gram-equivalent. Peroxide value is a ration of substance amount in a sample related to active oxygen, which under reference conditions oxidize potassium iodide, to the weight of studied sample. The value characterizes the amount of primary products of fats oxidation – peroxide compounds (hydroperoxides, peroxides, dialkyl peroxides), which are able to separate iodine from aqueous solution of potassium iodide. It is expressed in millimole of active oxygen per sample kilogram. Peroxide value is an indicator of fats freshness grade. The method principle is based on the interaction reaction of fat oxidation products with potassium iodide in acetic acid and chloroform solution and further quantitative determination of discharged iodine by sodium thiosulfate solution using titrimetric method.

Peroxide value (PV) 1/2 O mmol.kg^{-1} is calculated by formula:

$$PV = (V - V_0)*c*1000/ m \quad (1)$$

Where: V, V_0 – volume of sodium thiosulfate solution in main and control study respectively, cm^3; C – concentration of sodium thiosulfate solution, mol.dm^{-3}; m – mass of studied sample, g; 1000 – ratio, which includes conversion of measurement result in mmol.kg^{-1}.

Peroxide value is expressed in 1/2 O millimole per kilogram, which corresponds to the amount of oxygen, used in the given oxidation-reduction reaction, in milliequivalents per kilogram.

Determination of anisidine value was conducted under DSTU 6885-2002 **(DSTU 6885, 2002)**. The principle of the method is in dissolving the test sample of the fat product in isooctane, interacting with p-anisidine, and measuring the optical density of the solution at a wavelength of 350 nm. For determination of anisidine value such chemical reagent were used: sodium sulfate (Na_2SO_4) C.P.; 2,2,4-trimethylpentane (isooctane), which has optical density ≤0.01 in relation to water within the wavelength 300-380 nm; 4-methoxyaniline (p-anisidine); glacial acetic acid C.P., with content of water ≤0.1%; sodium sulphite (Na_2SO_3) C.P., anhydrous; activated carbon clarify powdered; anhydrous calcium chloride ($CaCl_2$) C.P.; laboratory items: spectrometer UV-1800 SHIMADZU, range of values 190 – 1100 nm, pathlength of cuvette 10 mm, suitable for measurement at the wavelength 350 nm. Anisidine value (AV) shows concentration of aldehydes (primarily, 2-alcanal) in animal fats. This is a dimensionless value, which is numerically equal to centuplicated optical density fat solution in isooctane upon processing by anisidine reagent, measured on spectrophotometer with a wavelength of 350 nm. The method principle consists in preparation of the studied sample solution in isooctane, reaction with acetic solution of p-anisidine and measurement of optical density at 350 nm.

Anisidine value is calculated by formula:

$$AV = 100 \cdot Q \cdot V [1.2 (A_1 - A_2) - A_0] / m, \quad (2)$$

Where: Q – adjusted concentration of studied solution in g per cm^3 (Q = 0.01 g.cm^{-3}); V – dissolved sample volume (V = 25 mL), A_0, A_1, A_2 – optical density of nonreactive analytical, color and control solution respectively; m – sample mass, g.

Integrated value of full fat oxidation (IV) is calculated by formula:

$$IV = 2\times PV+AV \quad (3)$$

Fat acidity is expressed in degrees c (°K), it is a volume (cm^3) of sodium or potassium hydroxide solution of molar concentration 0.1 mol.dm^{-3}, spent for neutralization of free acids in 10 g of product.

Statistical analysis

Obtained measurement results and graphical representation of experimental data were conducted using standard programs of statistical processing Microsoft Excel 2010. Precision of obtained results was ensured by three-, fivefold repetition of studies. The analysis of variance (ANOVA) of the obtained results was conducted; confidence range under t-criterion for parallel measurements with *p*-value less than 0.05 was determined. For building graphical dependencies and in the table the arithmetic results of parallel measurements with value of $p = 0.05$ are indicated.

RESULTS AND DISCUSSION

Since the peroxide value (PV) is regulated by state standards system for fat-containing products, there is a possibility to predict storage life of developed products under this value **(Coppin and Pike, 2001; Mehta et al., 2018)**. Generally accepted quality level of fat-containing products PV at the level up to 5.0 1/2 O mmol.kg^{-1}, when its value more than 10.0 1/2 O mmol.kg^{-1} the product loses its nutritional value **(Fatouh et al., 2005; Dyman and Zahoruy, 2008)**. Guaranteed storage life is established at the level of 50% of a term, during which the product does not lose its nutritional value, if expected storage life does not exceed 30 days and at the level of 70%, if expected storage life does not exceed 30 days **(Stele, 2006)**.

It was established that accumulation rate of peroxides during the storage of butter pastes directly depends on the storage temperature (Figure 2), caused by enzymic component of biochemical transformations of milk fat **(Jacobsen et al., 2008; Jones et al., 2005; Rodrigues and Gioielli, 2003)**. At temperature decrease the enzymes strength decreases and practically levels in condition of deep freezing (Figure 2a). It was determined that during storage at a temperature of (4 ±2 °C) (Figure 2a) within first 4 days, no major changes of peroxide value were observed, on further storage the accumulation rate of peroxides was rising ($p \leq 0.05$) significantly, on the 10^{th} day fat of butter pastes was defined by peroxide value higher than 5.0 1/2 O mmol.kg^{-1}, that was indicative of the beginning of spoilage processes, on the 15^{th} day of storage the peroxide value pf butter paste fat with milk and vegetable protein slightly exceeded permissible limits.

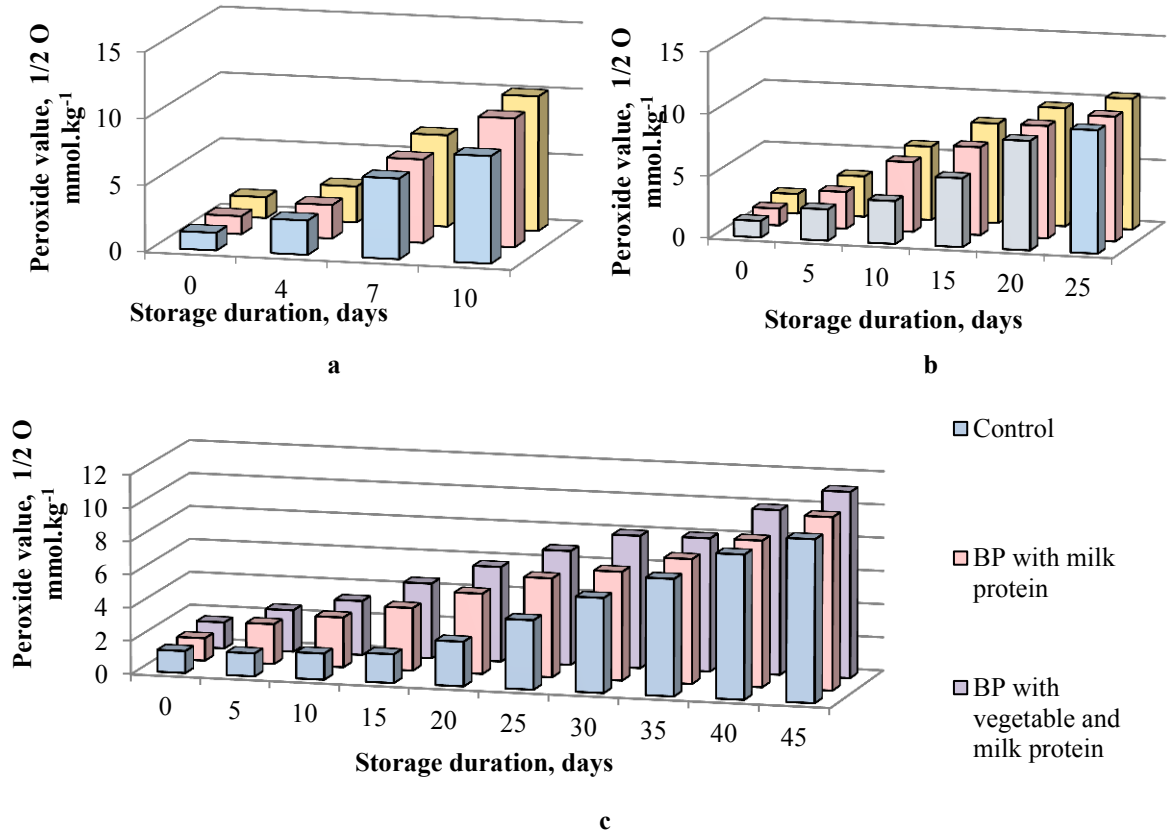

Figure 2 Dynamics of peroxide number of butter pastes at different temperature regimes: a – (4 ±2 °C); b – (-3 ±1 °C); c – (-24 ±2 °C); (n = 3 – 5, $p \leq 0.05$).

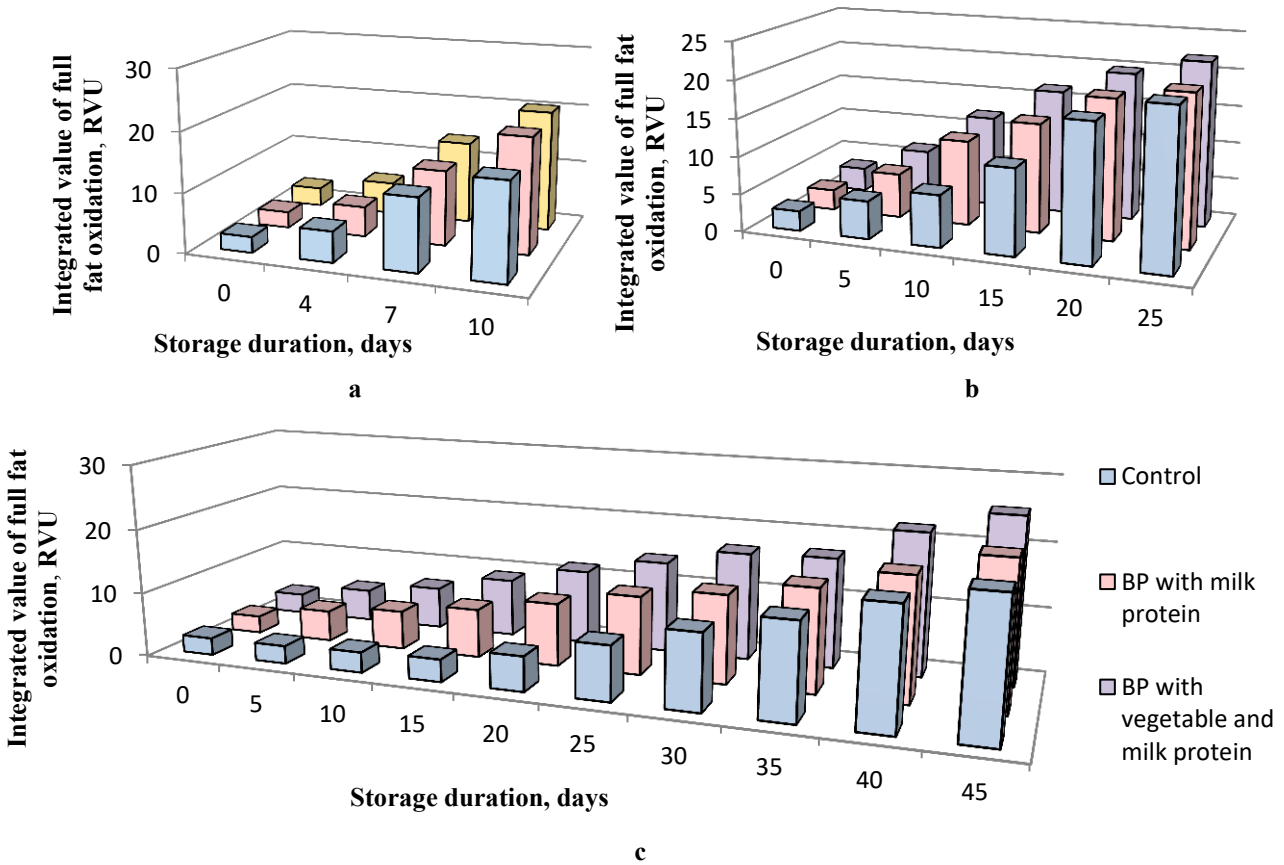

Figure 3 Dynamics of integral index of complete oxidation of butter pastes (relative value unit (RVU)) at different temperature regimes: a – (4 ±2 °C); b – (-3 ±1 °C); c – (-24 ±2 °C); (n = 3 – 5, $p \leq 0.05$).

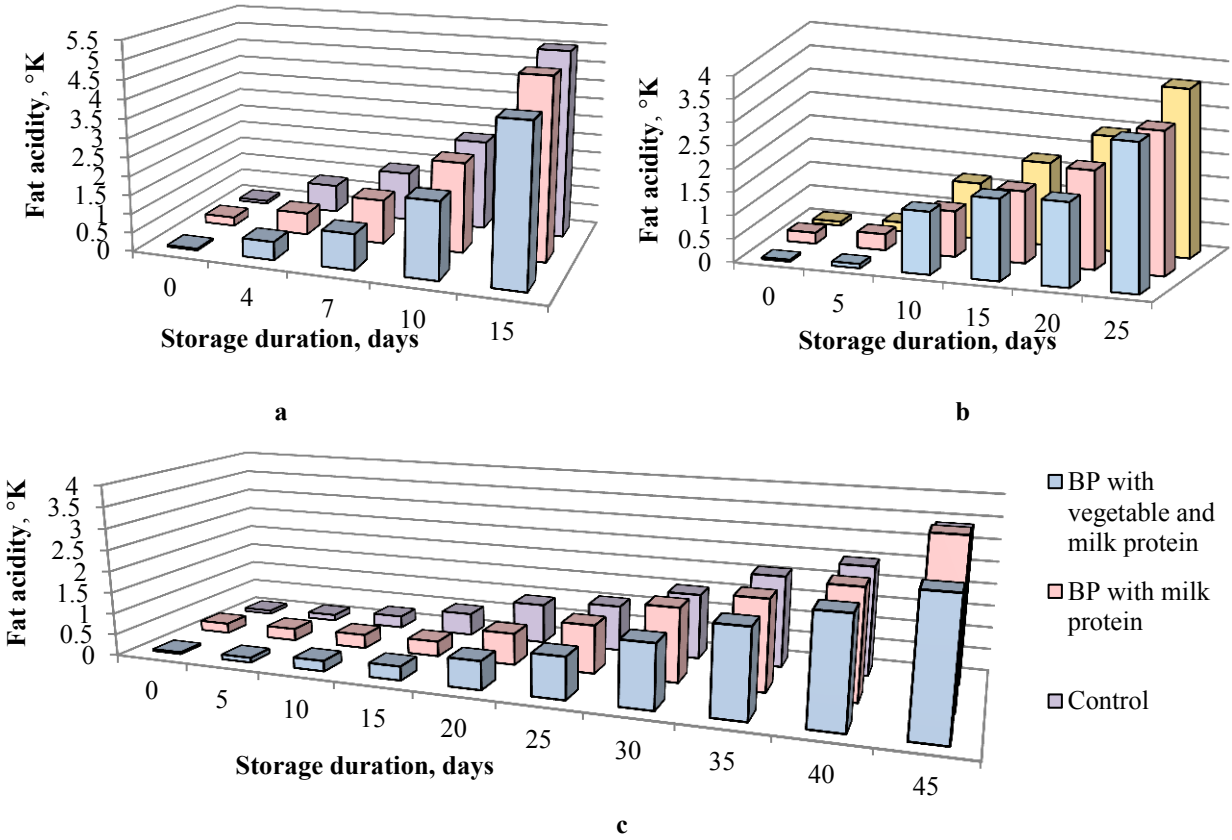

Figure 4 Acid dynamics of butter pastes at different temperature conditions: a – (4 ±2 °C); b – (-3 ±1 °C); c – (-24 ±2 °C); (n = 3 – 5, $p \leq 0.05$).

Based on obtained results we can conclude about the possibility of establishing guaranteed storage life – not more than 5 days. Accumulation rate of peroxides in butter pastes with vegetable protein is the highest among all studied samples in each particular control and observation point. The revealed trend can be explained by residual enzyme strength – lipooxidase, which is contained in vegetable isolate and catalyses reactions of lipid peroxide oxidation **(Lobanov and Shcherbin, 2003)**.

Similar trend was observed when studying peroxide value history at a temperature of (-3 ±1 °C) (Figure 2b). It was determined that butter paste fat could not be qualified as fresh when storing during 15 days at a said temperature, peroxide value exceeds permissible limits and is on average 10.4 1/2 O mmol.kg^{-1}. Thus, the guaranteed storage life of butter pastes at a said temperature should be maximum 15 days.

Rising dynamics of the peroxide value during storage of butter pastes at a temperature of (-24 ±2 °C) (Figure 2c) was far slower, peroxide value above 5 1/2 O mmol.kg^{-1} was detected on the 25th day of storage, exceeding of threshold value was observed on the 45th day of storage, allowing to establish guaranteed storage life of butter pastes for not more than 30 days at a temperature of (-24 ±2 °C).

The anisidine value is used to control processes of secondary oxidation by content of malondialdehyde. Substances with aldehyde functional groups are formed as a result of oxygen effect on peroxides, consequently they appear only at deep oxidation stages. When storing butter pastes at a temperature of (4 ±2 °C) secondary oxidation products were not detected. Under such storage conditions intense primary milk fat splitting takes place, resulting in product spoilage earlier that secondary oxidation products accumulate in it.

Obtained results show that AV at subzero temperatures does not exceed 1 c.u. value during the whole storage duration (Table 1). Aldehydes accumulation begins during the second half of storage in all studied temperature conditions.

Rising dynamics of full oxidation of butter pastes, stabilized by protein polysaccharide complexes based on milk and vegetable protein, was faster as compared to control. It is explained by the presence of fat-soluble emulsifiers in the formulation of butter paste as a reference product **(Lucas et al., 2010)** (Figure 3) that have a protective effect on milk fat glycerides. Fat splitting process in butter pastes with protein polysaccharide complexes takes place with almost the same intensity (due to the influence of milk protein concentrate, **(Abdullah et al., 2018)**).

Fat breakdown with formation of fatty acids can assist in acceleration of oxidation process because free acids oxidize first. Total amount of acidic substances in the product is indirect indicator of hydrolytic fat decomposition during the product storage and is characterized by acid value **(Musiy and Tsisaryk, 2014)**. Besides, acidity of butter pastes is stipulated by the

presence of organic acids, formed during the breakdown process of plasma lactose, by acidic products of proteins breakdown etc. **(Hladyshev, 2012; Malfia et al., 2008)**. Results depicted on Figure 4 show that acid value of developed butter pastes is credibly lower than acidity of reference sample. Accumulation of organic acids takes place slower in butter pastes with protein polysaccharide complex under all studied temperature conditions regardless of freezing depth **(Ruttarattanamongkol, Afizah and Rizvi, 2015)**.

Thus, acid value did not exceed recommended limits (2.5 °K) and was on average – 2.3 °K when storing butter paste during 10 days at a temperature of (4 ±2 °C); 2.1 °K during 20 days at a temperature of (-3 ±1 °C), 2.4 °K during 40 days at a temperature of (-24 ±2 °C). Slightly lower acid values of milk fat of reference sample can be similarly explained by protective effect on lipid of formulation components – fat-soluble emulsifier **(Bodnarchuk, 2016)**.

CONCLUSION

It was established that storage of butter pastes at above-zero temperatures causes intensive milk fat splitting forming primary splitting products – peroxides that results in product spoilage. Research results of milk fat oxidation processes of new butter paste types stabilized by protein polysaccharide complexes and reference sample during storage at below zero temperatures showed common trends. At first accumulation of primary and secondary oxidation products takes place slower, upon which jumping increase at the expense of organic acids accumulation was observed that causes intensification of oxidation processes. Based on the obtained evaluation results of fat phase stability of studied butter pastes, the following storage life is recommended: at a temperature of (4 ±2 °C) during maximum 7 days, at a temperature of (-3 ±1 °C) during maximum 15 days, at a temperature of (-24 ±2 °C) during maximum 30 days. Research results and found regularities will be used for complex evaluation of quality and stability of new butter paste types, stabilized by protein polysaccharide complexes for the purpose of justification of their guaranteed storage life.

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THE INFLUENCE OF CAVITATION EFFECTS ON THE PURIFICATION PROCESSES OF BEET SUGAR PRODUCTION JUICES

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ABSTRACT
The existing technology for the purification of diffusion juice and its hardware design has not fundamentally changed over the past four decades. The lack of the necessary theoretical developments and experimental data hinders the development of existing and the development of new technological processes. Therefore, the main direction of improving the purification efficiency of juices of sugar beet production is the disclosure of its unused reserves and their implementation in practice. The scientific problem of choosing the rational direction for improving the technology of juice purification, which ensures the production of consumer granulated sugar in the face of changes in a wide range of quality of raw materials, is relevant and has important economic importance, especially in the context of the transition of beet sugar factories to a market economy. One way to solve it is to make fuller use of the adsorption capacity of calcium carbonate particles while increasing the filtration properties of saturation sediments. Therefore, the study investigates the effect of cavitation effects – vapor condensation and hydrodynamic processing of diffusion juice on the processes of purification of diffusion juice, juices of preliminary defecation, first and second saturations. The analysis of the influence of various effects of cavitation processing of juices from the point of view of improving the purification efficiency, the optimal place of the purification process in the technological scheme of production is established.

Keywords: diffuse juice; preliminary defecation; saturation; intensification; cavitation

INTRODUCTION
Classical methods of intensifying the processes of calc-carbon dioxide purification of diffusion juice of sugar beet production by changing the temperature, alkalinity, or duration of individual stages do not provide an increase in the purification efficiency. In particular, analyzing the conditions of the processes at the previous defecation is found **(Ozerov and Sapronov, 1985; Johnson, Zhou and Wangersky, 1986)** that the limiting factor at this stage is the rate of delivery of reagents to the reaction zone. In this regard, the authors of **(Lee et al., 2012)** propose a method of intensifying preliminary bowel movements using hydrodynamic (HD) cavitation, which significantly accelerates the mixing of reagents due to shock-wave action during the collapse of cavitation bubbles. But under these conditions, along with positive signs, there is also a slight deterioration in the sedimentation-filtration properties of juice of I saturation **(Almohammed, et al., 2015; Palamarchuk et al., 2019)**, which can most likely be explained by cavitation destruction (grinding) of sediment particles formed during the previous defecation.

There is also information about the application of the method of blowing steam into the juice stream to intensify the purification and sedimentation processes **(Lebovka, et al., 2007; Dalfré Filho, Assis and Genovez, 2015)**, which helps to reduce the content of calcium salts and the color of the juice of II saturation **(Kim et al., 2016)**.

But in the mentioned works (especially on the injection of water vapor), the physical essence of the effects and the mechanisms of their action on the physicochemical transformations of non-curved diffusion juice is not considered, but only the positive consequences taking place, in this case, are given. In addition, the literature does not contain data comparing the effects of hydrodynamic (HD) or vapor condensation (VC) cavitation processing of juices in terms of improving the purification efficiency and research to establish the optimal place in the technological scheme of purification and has become the subject of our research.

Scientific hypothesis
The scientific hypothesis lies in the rational direction of the intensification of the technology for the purification of beet sugar juices. It is assumed that an increase in the quality and yield of sugar is possible due to the collapse of steam bubbles during the vapor condensation and

hydrodynamic cavitation. The latter accelerate the main reactions at the stages of juice purification, leading to an increase in the overall purification effect.

MATERIAL AND METHODOLOGY

The objects of research are diffuse juice, juice of preliminary defecation, juice of the first and second saturation. Diffuse juice is processed in a hydrodynamic cavitation installation under optimal conditions. Samples of diffusion juice according to standard methods **(Ozerov and Sapronov, 1985)** in laboratory conditions are processed before the juice of preliminary defecation and the first and second saturation.

For preliminary defecation juice and juice of the first saturation, the sedimentation rate and juice sedimentation volume after settling are determined after 20 minutes; for the second saturation juice, the content of calcium salts in percent by weight of the juice and its purity are determined.

It is known that the shock-wave effect of a hydrodynamic cavitation field on the medium is processed, determined by the stage of cavitation **(Sheiko et al., 2019; Sukhenko et al., 2019)**. In this case, the effective regime of cavitation mixing and dispersion corresponds to the maximum of the shock-wave action of cavitation field bubbles on the medium, it is being processed **(Matyashchuk et al., 1988)**. Therefore, the cavitation stage λ is used by us as a determining parameter for characterizing the operation mode of a hydrodynamic cavitation device and ranged from 0.62 to 4.0. This choice is because at λ = 1.0 a flow regime arises, which is characterized by a bubble form of cavitation, which can be considered transitional from turbulent to cavitation. At λ = 4.0, which is characterized by a mixed form of cavitation, a super-cavitation regime begins to form.

To determine the sedimentation rate, a 2-liter cylinder was used, which was used to determine the height of the clarified suspension and the sedimentation rate after 5 minutes. To determine the purity of the juice it is necessary: to divide the sucrose content by the dry matter content. The dry matter content was determined using a URL-1 refractometer (supplier company PrimaRia, Kiev, Ukraine), and the sucrose content was determined using a SU-4 saccharometer (supplier company PrimaRia, Kiev, Ukraine).

Processing of the medium in the working area of the hydrodynamic cavitation installation occurs under the influence of cavitation bubbles, splashing. Therefore, the number and size of the formed cavitation bubbles are the determining factors of the technological efficiency of the cavitation treatment. In turn, the structure of the field of cavitation bubbles depends on the hydrodynamic parameters of the process, the main of which are the flow rate in the gap between the cavitator and the wall of the working section of the cavitation device and the stage of cavitation λ **(Luo et al., 2019)**. Therefore, the establishment of the hydrodynamic operating conditions of the installation was first carried out on barometric water with a temperature of 50 °C, which is similar for diffusion juice (diffusion column). The compression ratio of the flow in the working area of the laboratory setup is changed by establishing cavitators of different diameters with a Reynolds number of (23.6 – 25.4) $x10^4$. The cavitation stage was also determined on the water by visual measurement of the cavitation length in its characteristic radius.

Statistic analysis

Mathematical and statistical processing of experimental data was carried out in determining the criteria of Cochran's C test, Fisher and Student's *t*-test. The accuracy of the data was determined using the Cochrane criterion, and the adequacy of the mathematical model was checked using the Fisher and Student criteria. Statistical processing was performed in Microsoft Excel 2013 values were estimated using mean and standard deviations.

RESULTS AND DISCUSSION

In **Kozelová et al. (2011)** and **Zheplinska et al. (2019)** it is found that the efficiency of processing diffusion juice in a hydrodynamic cavitation device with increasing compression ratio increases and is largest at $d_{к\text{-}ра}/d_p$ = 0.8. Therefore, the effect of the cavitation stage on juice processing is determined at a given constant value of the compression ratio **(Jia-Qian, 2015)**. Figure 1 shows the technological parameters of the juice of preliminary defecation during the processing of diffusion juice in the HD cavitation device with a compression ratio of 0.8.

As can be seen from Figure 1 the best technological indicators of juice when cutting diffusion juice during the cavitation stage 2.3. This is indicated by the maximum deposition rate and the smallest amount of sediment juice pre-defecation. Therefore, the cavitation stage, equal to 2.3, is chosen for processing diffusion juice to determine the effect of cavitation effects on subsequent juice purification processes. In the work of the authors **(Somaratne et al., 2019; Shmyrin, Kanyugina and Kuznetsov, 2017)** the stage of cavitation 3.3 was chosen, which in our opinion will lead to a decrease in the yield of the main product.

Comparative characteristics of diffusion juice samples, the purifications of which are carried out according to the standard scheme and according to the scheme with preliminary processing of diffusion juice in a hydrodynamic cavitation device, are given in Table 1.

As can be seen from the experimental data, cavitation processing of diffusion juice intensifies the processes of coagulation of substances of a colloidal dispersion of diffusion juice at the previous defecation and adsorption of non-curvatures at the stages of the first and second saturation, as evidenced, in particular, by an increase in the purity of purified juice by 0.7%. The authors of **(Bhatia et al., 2016)** concluded that cavitation treatment of diffusion juice intensifies the processes and leads to an increase in the purity of purified juice by only 0.3%, and the authors of **(Verma et al., 2018)** purity of purified juices on the contrary decreases by 0.1%

To determine in the technological scheme, the most appropriate place for the hydrodynamic cavitation processing of sugar production juices, let's carry out studies in which juices: diffuse, preliminary defecation, and main defecation are cleared in the hydrodynamic cavitation installation. Samples of juices after a single treatment from the cavitation stage λ = 2.3 in laboratory conditions are brought to the juice of the first saturation and S_5 and V_{20} re determined in them. Figure 2 presents the values of these

values for each of the juices. As can be seen from the research results, the best indicators of the juice of the first saturation according to the scheme when the diffusion juice is processed in the hydrodynamic cavitation device.

In this case, the deposition rate during the processing of diffusion juice in a hydrodynamic cavitation device compared with the conventional method increases by 19.4%, and the sediment volume decreases by 14%. When processing juices of preliminary and main defecation, the technological properties of the juice of the first saturation significantly (confidence probability $p = 0.95$).

deteriorate in comparison with the usual purification method. Similar results were obtained in studies presented in the works of the following authors **(Katariya, Arya and Pandit, 2020; Krasulya et al., 2016).**

Processing in the installation of juices of preliminary and main bowel movements leads to a slight deterioration in the indicators of the juice of the first saturation – a decrease in the sedimentation rate of juice by 6 and 12.5%, respectively, and sediment volume by 7 and 20.7% compared with the usual method of juice purification. This indicates that during the processing of juices of preliminary and main defecation, the sediment particles formed at the stage of preliminary defecation are destroyed, followed by hydration of the substances of colloidal dispersion (SCD) particles that are adsorbed on it. Due to this, the deposition rate decreases, and the volume of juice sediment after settling increases. Similar data were obtained in work **(Guo et al., 2018)**, in which the juice of preliminary defecation was processed in a cavitation apparatus with a cavitator of a dynamic type before the main defecation, where the milk of lime is fed **(Dhar et al., 2015)**.

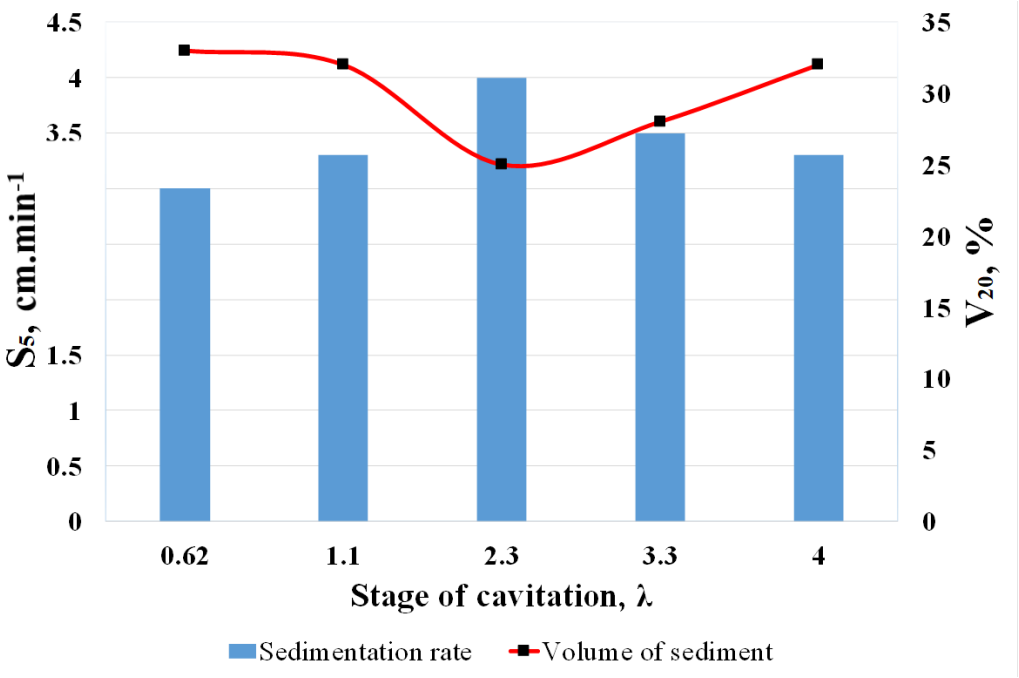

Figure 1 The effect of the cavitation stage on the technological parameters of preliminary defecation juice.

Table 1 Technological indicators of juices at diffusion juice purification according to a standard scheme and using the effects of HD cavitation.

Purification scheme	Juice						
	Diffuse	Preliminary defecation		First saturation		Second saturation	
	Purity, %	S_5, cm.min^{-1}	V_{20}, %	S_5, cm.min^{-1}	V_{20}, %	Salts Ca^{2+}, % CaO	Purity,%
Typical	86.5	3.2	33	3.3	30	0.04	90.3
	86.3	3.0	35	3.1	31	0.036	90.5
	86.7	3.3	32	3.3	31	0.04	90.6
Average	86.5	3.2	33.3	3.2	30.7	0.039	90.9
With hydrodynamic cavitation	86.5	3.7	32	3.9	25	0.021	91.3
	86.3	3.9	30	4.1	26	0.020	91.5
	86.7	4.0	30	4.1	24	0.021	91.5
Average	86.5	3.9	29.0	4.0	25.0	0.021	91.6

Note: * all studies were performed in 5 replicates, the deviation of which did not exceed 3%.

Table 2 Comparative characteristics of technological indicators of juices at diffusion juice purification according to a standard scheme and using the effects of HD and VC cavitation.

Purification scheme	Juice							
	Diffuse		Preliminary defecation		First saturation		Second saturation	
	Δt, °C	Purity, %	S_5, cm.min^{-1}	V_{20}, %	S_5, cm.min^{-1}	V_{20}, %	Salts Ca^{2+}, % CaO	Purity, %
Typical	–	86.5	3.2	33	3.3	30	0.04	90.3
	–	86.3	3.0	35	3.1	31	0.036	90.5
	–	86.7	3.3	32	3,3	31	0.04	90.6
Average	–	86.5	3.2	33.3	3.2	30.7	0.039	90.9
With processing in a VC cavitation device	4	86.5	3.5	29	3.7	26	0.025	91.1
	5	86.3	3.7	30	3.9	27	0.021	91.3
	4	86.7	3.8	28	4.1	25	0.027	91.0
Average	4.3	86.5	3.7	29.0	3.9	26.0	0.028	91.4
With processing in a HD cavitation device	–	86.5	3.7	32	3.9	25	0.021	91.3
	–	86.3	3.9	30	4.1	26	0.020	91.5
	–	86.7	4.0	30	4.1	24	0.021	91.5
Average	–	86.5	3.9	29.0	4.0	25.0	0.021	91.6

Note: *all studies were performed in 5 replicates, the deviation of which did not exceed 3%.

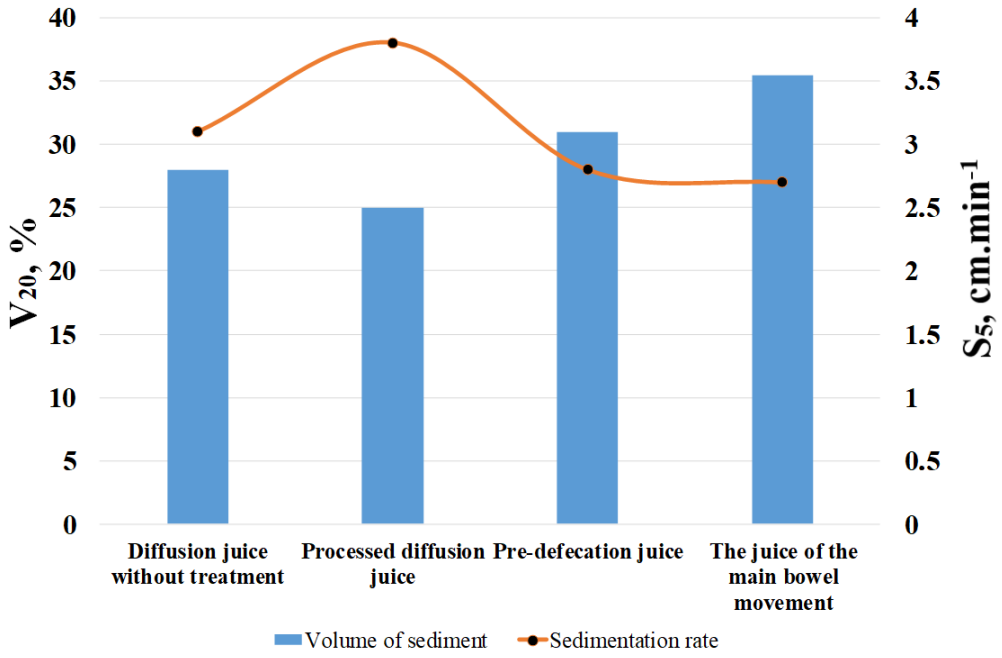

Figure 2 Technological indicators of the juice of the first saturation in the processing of juices of the main unit

The authors found improvements in the physicochemical parameters of the juices of the first and second saturation.

Such changes are explained by the fact that the activity of Ca^{2+} ions is increased, which are released from the clathrate structure of the hexo aqua complex, which interacts more fully with non-sugars juice. However, the sedimentation rate of the juice of the first saturation decreases by 10%, and the filtration coefficient increases.

From an analysis of laboratory studies, it can be assumed that the cavitation treatment of diffusion juice in a hydrodynamic cavitation device is the first step in the conversion of particles of substances of a colloidal dispersion of juice. After a certain period of time, they interact with each other, or with a chemical reagent, if any, which will contribute to the formation of a compact, low-hydrated coagulate precipitate during the previous defecation, which is resistant to peptization under conditions of basic defecation. As a result, the filtration properties of juice and carbonation will improve and the effect of the juice of I purification as a whole will increase.

To establish an analogy of the effect of the studied effects on the purification of diffusion juice in cavitation devices, parallel experiments are conducted in which the diffuse juice after treatment in hydrodynamic and vapor condensation cavitation devices is cleaned according to the following scheme: optimal previous defecation at a

temperature of 55 – 65 °C, which is fed 0.25 – 0.3% CaO at pH_{20} 10.8 – 11.2, heating to a temperature of 80 – 85 °C, the main defecation with the addition of 2.5% CaO, the first saturation to pH_{20} 10.8 – 11.2, sediment separation of the juice of first second saturation by decantation followed by filtration; second saturation; filtering the juice of the second saturation.

Comparative values of juices according to the standard scheme and with cavitation processing of diffusion juice are given in Table 2.

According to research results, when processing diffusion juice in cavitation devices, the performance of juices is improved compared to the usual purification method. So, the sedimentation rate of preliminary defecation juice after processing diffusion juice in a vapor condensation cavitation device increases by 15.6%, and in a hydrodynamic cavitation device by 21.8%, the volume of juice sediment decreases in both cases by 12.9%. A similar dependence is observed in the juice of the first saturation: the sedimentation rate of the juice sediment processed in the vapor condensation cavitation device increases accordingly by 21.8%, in the hydrodynamic cavitation device - by 25%, the volume of juice sediment decreases by 15.3 and 18, 6, respectively % The same dependence remains with the content of calcium salts in the juice of the second saturation. According to calcium salts are reduced by 28.2 and 46.2%.

The authors **(Buniowska et al., 2017; Alves et al., 2017)** conducted similar studies only without the use of cavial influence and other temperature characteristics, the volume of juice precipitate decreases by only 8.3 and 9.6%.

Purification is carried out after sampling under the action of gravitational forces in production conditions, ie the process of deposition (settling). The difference in the results is that the samples were taken at the existing sugar factory directly in the production conditions in the stream, so the possibility of their variation depends on the technological parameters, which may vary slightly.

CONCLUSION

From comparisons of the technological indicators of juices, it can be concluded that upon the collapse of steam bubbles in vapor condensation and hydrodynamic cavitation devices, structural transformations of non-curved diffusion juice occur. This means that during the collapse of bubbles in vapor condensation and hydrodynamic cavitation devices, similar effects occur that positively affect the efficiency of juice purification. However, the indicators of juices processed in a hydrodynamic cavitation device are somewhat better than in the vapor condensation one. This indicates, in our opinion, that the distribution of bubbles in the hydrodynamic cavitation device is more uniform than in the vapor condensation one due to the fact that the flow velocity in the hydrodynamic cavitation device is higher, and, as a consequence, the flow turbulence is higher. It is established that the cavitation treatment of diffusion juice before liming causes a significantly (p = 0.95) acceleration of the course of the main reactions to the previous and main bowel movements and an increase in the overall purification effect. But the mechanism of the influence of cavitation phenomena on the components of diffusion juice remains unknown and will be the subject of our further research since without this it is impossible to improve or optimize the processes of sugar beet production.

The proposed purification scheme allows compared to the typical scheme to reduce the content of lime milk for the second saturation by 0.018% CaO (almost 2 times), accelerate the process of separating the solid phase from the liquid (settling), as evidenced by the volume of juice and sedimentation rate. are better by 3.4 and 2.5%, respectively, and increase the purity of the juice by 0.7 units. And this will increase the yield of sugar.

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A RANDOMISED CONTROLLED TRIAL OF INNOVATIVE SPECIALISED MEAT PRODUCT FOR PATIENTS WITH CARDIOVASCULAR AND METABOLIC DISORDERS

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ABSTRACT

Cardiovascular diseases remain one of the leading causes of death globally. A lot of dietary patterns for CVD prevention have been proposed, but special attention is paid to functional foods. Bioactive proteins and peptides from animal sources are also considered tools for the prevention of CVDs. Here, 40 overweight or obese adult men and women aged between 61 and 66 years, with a body-mass index between 28 and 61 $kg.m^{-2}$, were enrolled into a randomised controlled trial of new meat products for specialised nutrition. Participants in the control group (n = 20) consumed a standard hyponatric low-calorie diet for 28-30 days (10 days inpatient and 18-20 days outpatient), and in the experimental group – a low-calorie diet and 100g developed meat product (ratio of the porcine aorta to hearts 1:3) per day. Total cholesterol, triglyceride, cholesterol low-density lipoprotein, and cholesterol high-density lipoprotein levels were measured in the serum; from this, the atherogenic index was calculated. The positive effect of developed meat products on the serum lipid profile of patients during the trial was mild but noticeable. A significant reduction in cholesterol levels was noticed in the experimental group, by 18.2% and 14.0% after 7 – 10 and 28 – 30 days, respectively, while the cholesterol level in the control group returned to its original level after 28 – 30 days of dieting. The difference between the control and experimental groups was not significant, while data in the percentiles were. Therefore, it is more preferable to use a developed product as a component in diet therapy for hyperlipidaemic humans for over 28 – 30 days. Pronounced effects of the product could be linked to the unique proteome and peptidome of heart and aorta tissues based on organ-specific gene expression and the presence of tissue-specific substances.

Keywords: CVD; metabolic disorder; functional food; hyperlipidemia; cholesterol

INTRODUCTION

According to World Health Organisation data, cardiovascular diseases (CVDs) are the leading cause of death globally, taking an estimated 17.9 million lives each year. An unhealthy diet is one of the main risk factors for CVD, as well as high blood cholesterol and high blood sugar or glucose levels. Therefore, CVDs are often accompanied by metabolic syndrome and diabetes **(Han and Lean, 2016; Saklayen, 2018)**.

A lot of dietary patterns for the prevention of CVDs have been proposed, such as low-fat diets enriched with monounsaturated (MUFA) and polyunsaturated (PUFA) fats, low-carbohydrate diets enriched with fibres, the Dietary Approach to Stop Hypertension (DASH) diet providing more calcium, potassium, magnesium, and dietary fibre and less fat, saturated fatty acids (SFA), cholesterol, and sodium, and the Mediterranean diet characterised by a relatively high fat intake (40–50% of total daily calories), of which SFA comprises ≤8% and MUFA 15–25% of calories **(Eilat-Adar et al., 2013; Anand et al., 2015; Mozaffarian, 2016)**.

Composition modification (fatty acids modification, sodium chloride control), fermentation (formation of bioactive compounds) and the introduction of functional additives (plant components (oils, extracts, fibres), soy protein, natural and synthetic antioxidants, lactic acid bacteria, and fish oil are successfully used in functional food processing **(Mine and Shahidi, 2006; Cencic and Chingwaru, 2010; Alissa and Ferns, 2012; Hui, 2012; Griffiths et al., 2016; Lordan et al., 2018)**.

Peptides of animal origin released from milk and meat proteins during proteolysis, fermentation or food processing possess hypotensive, antioxidant, antimicrobial, antitumor, antithrombotic, lipid-lowering, and opioid *etc.* actions, and can be considered functional additives to food **(Arihara, 2006; Ahhmed and Muguruma, 2010; Ryan et al., 2011; Udenigwe and Howard, 2013; Lafarga and Hayes, 2014; Bhat, Kumar and Bhat, 2015; Liu et al.,**

2016; Sánchez and Vázquez, 2017). However, tissue-specific proteins and peptides contained natively in slaughter by-products have still not been well-studied.

In previous studies, we confirmed the lipid-lowering effect of functional meat products derived from the porcine heart and aorta on hyperlipidaemic rats **(Chernukha et al., 2018; Kotenkova and Chernukha, 2019)**. This paper reports the analytical results of a randomised controlled trial of meat products for specialised nutrition in patients with cardiovascular and metabolic disorders.

Scientific hypothesis

A wide range of tissue-specific proteins and peptides were identified in raw porcine heart and aorta, some of which were decomposed during meat product processing. The hypothesis that tissue-specific proteins could decompose into active peptides with similar biological action was proven in hyperlipidaemic rats that consumed the innovative product.

Nevertheless, the effectiveness of the product on rodents and humans can vary significantly; therefore, the hypolipidaemic action was studied during a randomised controlled trial on patients with cardiovascular and metabolic disorders.

MATERIAL AND METHODOLOGY

Meat products (MsP) for specialised nutrition were produced on ZAO "Yoshkar-Olinskiy Myasokombinat". Porcine hearts were chopped with a particle size of 2 – 3 mm and salted for 12 h. Porcine aortas were chopped with a particle size of 2 – 3 mm and homogenised in a cutter at 3000rpm for 2 – 3 min. Minced hearts with the juice were quantitatively transferred in the cutter and homogenised at 3000rpm for 6 – 8 min (ratio of the aorta to hearts 1:3). The obtained mince was packed in cans of lamister and sterilised at 115 °C and a pressure of 0.23 MPa for 40 min. Meat products contained 17.53 ±0.95% protein, 3.82 ±0.13% fat, 0.305 ±0.015% sodium chloride, and 2.35 ±0.25% starch.

Study design

The open, prospective, and randomised study was conducted based on the Department of Cardiovascular Pathology of the Federal Research Centre of Nutrition and Biotechnology from June 01, 2019, to October 15, 2019.

The study protocol was approved by the Ethics Committee of the Federal Research Centre of Nutrition and Biotechnology (Protocol No. 7 of 03.12.2018). In accordance with the GCP program, all participants signed written informed consent.

Randomization and study groups

Forty overweight or obese adult men and women aged between 61 and 66 years, with a body-mass index between 28 and 61 kg.m^{-2}, were enrolled.

Patients were randomised into two groups by flipping a coin. The participants were not blinded to their group assignment. Participants in the control group (n = 20) consumed a standard hyponatric low-calorie diet (LCD) for 28 – 30 days (10 days inpatient and 18 – 20 days outpatient), and in the experimental group received LCD and 100g MP per day.

Dietary intake

LCD is a diet with a significant restriction of fat and easily digestible carbohydrates, normal protein, and complex carbohydrates, with an increased amount of dietary fibre and a reduction of table salt (3 – 5 g/day). Dishes are boiled, stewed, baked, pureed, and not pureed, as well as steamed. The food temperature ranged from 15 °C to 60 – 65ºC, while the free liquid is 0.8 – 1.5 L. Nutrition is fractional, with addition 4-6 times a day.

Comparative characteristics of the chemical composition of LCD and modified diet with the inclusion of MP are presented in Table 1.

Biochemical analysis

Blood samples for biochemical studies were taken on 0, 7 – 10 and 28 – 30 days. Biochemical investigations were carried out on an automatic analyser BioChem FC-360 (HTI, USA) according to the instructions supplied with the measurement kits (HTI, USA). Total cholesterol (TCL), triglyceride (TG), cholesterol low-density lipoprotein (CL LDL), and cholesterol high-density lipoprotein (CL HDL) levels were measured in serum. Atherogenic index (AI) = (TCL - CL HDL)/CL HDL.

Statistical analysis

STATISTICA 10.0 software was used in this study for the statistical analyses. The results were calculated as "middle value ± standard deviation" ($M \pm SD$) and "percentile" ($P_{25\backslash 75}$). Significant differences were tested by nonparametric statistical Mann-Whitney U tests for independent variables and Freidman ANOVA for dependent variables. Differences with p-values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

The null hypothesis about the influence of control and experimental diets on serum lipid profile inside the group were checked according to Freidman's ANOVA for all patients and patients with a BMI below 40 kg.m^{-2} ($n_{control}$ = 11, $n_{experimental}$ = 10) and above 40 kg.m^{-2} ($n_{control}$ = 9, $n_{experimental}$ = 10). The results are presented in Table 1. It was shown that LCD consumption (control) for 7 – 10 days led to total cholesterol reduction by 11.2%, mainly due to its reduction in the serum of patients with a

Table 1 Comparative characteristics of the chemical composition of LCD and modified diet with the inclusion of MP.

Diet	Chemical composition			
	Energy value, kcal/day	Proteins, g/day	Fat, g/day	Carbohydrates, g/day
LCD	1350.0 – 1550.0	70.0 – 80.0	60.0 – 70.0	130.0 – 150.0
LCD+MP	1437.0 – 1682.0	86.5 – 98.0	63.0 – 76.0	131.0 – 151.5

Table 1 Serum lipid profile of patients during trial.

Groups	0 days	7 – 10 days	28 – 30 days	*p*-value (Freidman ANOVA)
Total Cholesterol, mmol.L^{-1}				
Control group				
Total patients	3.829 ±0.777	3.400 ±0.695	4.025 ±1.225	0.1423
Patients with BMI >40 kg.m^{-2}	3.600 ±0.817	3.513 ±0.773	3.860 ±1.386	0.7165
Patients with BMI <40 kg.m^{-2}	4.016 ±0.726	3.307 ±0.647	4.160 ±1.127	0.1496
Experimental group				
Total patients	4.637 ±1.411	3.791 ±1.109	3.989 ±0.955	**0.0008**
Patients with BMI >40 kg.m^{-2}	4.474 ±1.564	3.202 ±0.606	3.901 ±0.839	**0.0018**
Patients with BMI <40 kg.m^{-2}	4.800 ±1.303	4.381 ±1.208	4.078 ±1.097	0.0672
Cholesterol LDL, mmol.L^{-1}				
Control group				
Total patients	1.981 ±0.535	1.708 ±0.469	2.241 ±1.008	**0.0174**
Patients with BMI >40 kg.m^{-2}	2.041 ±0.479	1.769 ±0.459	2.296 ±1.193	0.3679
Patients with BMI <40 kg.m^{-2}	1.931 ±0.596	1.658 ±0.493	2.196 ±0.888	**0.0379**
Experimental group				
Total patients	2.691 ±1.151	2.089 ±0.884	2.230 ±0.697	**0.0024**
Patients with BMI >40 kg.m^{-2}	2.517 ±1.264	1.601 ±0.466	2.093 ±0.617	**0.0018**
Patients with BMI <40 kg.m^{-2}	2.864 ±1.064	2.576 ±0.951	2.367 ±0.777	0.1496
Cholesterol HDL, mmol.L^{-1}				
Control group				
Total patients	1.145 ±0.286	1.131 ±0.269	1.191 ±0.245	**0.0150**
Patients with BMI >40 kg.m^{-2}	0.965 ±0.297	1.066 ±0.342	1.028 ±0.231	0.1211
Patients with BMI <40 kg.m^{-2}	1.291 ±0.179	1.185 ±0.192	1.323 ±0.169	**0.0116**
Experimental group				
Total patients	1.118 ±0.258	1.016 ±0.258	1.155 ±0.262	**0.0106**
Patients with BMI >40 kg.m^{-2}	1.147 ±0.283	1.015 ±0.201	1.193 ±0.278	**0.0055**
Patients with BMI <40 kg.m^{-2}	1.090 ±0.242	1.016 ±0.178	1.117 ±0.254	0.4966
Triglycerides, mmol.L^{-1}				
Control group				
Total patients	1.039 ±0.433	1.000 ±0.452	1.098 ±0.493	0.2466
Patients with BMI >40 kg.m^{-2}	1.193 ±0.541	1.157 ±0.404	1.147 ±0.492	0.8948
Patients with BMI <40 kg.m^{-2}	0.913 ±0.290	0.872 ±0.465	1.058 ±0.514	0.1482
Experimental group				
Total patients	1.919 ±0.987	1.689 ±0.777	1.585 ±0.866	0.3499
Patients with BMI >40 kg.m^{-2}	1.760 ±0.781	1.600 ±0.833	1.341 ±0.482	0.9048
Patients with BMI <40 kg.m^{-2}	2.078 ±1.179	1.778 ±0.751	1.829 ±0.104	0.2725
Serum atherogenic index				
Control group				
Total patients	2.494 ±0.798	2.085 ±0.628	2.465 ±1.048	0.0863
Patients with BMI >40 kg.m^{-2}	2.924 ±0.837	2.398 ±0.603	2.830 ±1.225	0.1211
Patients with BMI <40 kg.m^{-2}	2.141 ±0.587	1.828 ±0.545	2.167 ±0.817	0.1777
Experimental group				
Total patients	3.192 ±0.968	2.756 ±0.936	2.540 ±0.864	0.0863
Patients with BMI >40 kg.m^{-2}	2.959 ±1.107	2.190 ±0.503	2.352 ±0.707	0.2725
Patients with BMI <40 kg.m^{-2}	3.425 ±0.795	3.320 ±0.941	2.728 ±0.999	0.2019

Note: p <0.05 mean that diet consumption influence on lipid parameter in serum.

BMI below 40 kg.m^{-2} by 17.7%.

The cholesterol level returned to its original level after 28–30 days of dieting. Revealed changes were not significantly reliable. LCD with MP consumption (experimental) led to a statistically significant total cholesterol reduction by 18.2% and 14.0% after 7 – 10 and 28 – 30 days, respectively, mainly due to its reduction in the serum of patients with a BMI above 40 kg.m^{-2} by 28.4% and 12.8%, respectively. Despite the lack of statistical significance, it was noted that the total cholesterol level reduction was higher in patients with a BMI below 40 kg.m^{-2} and equal to 15.0% compared with day 0.

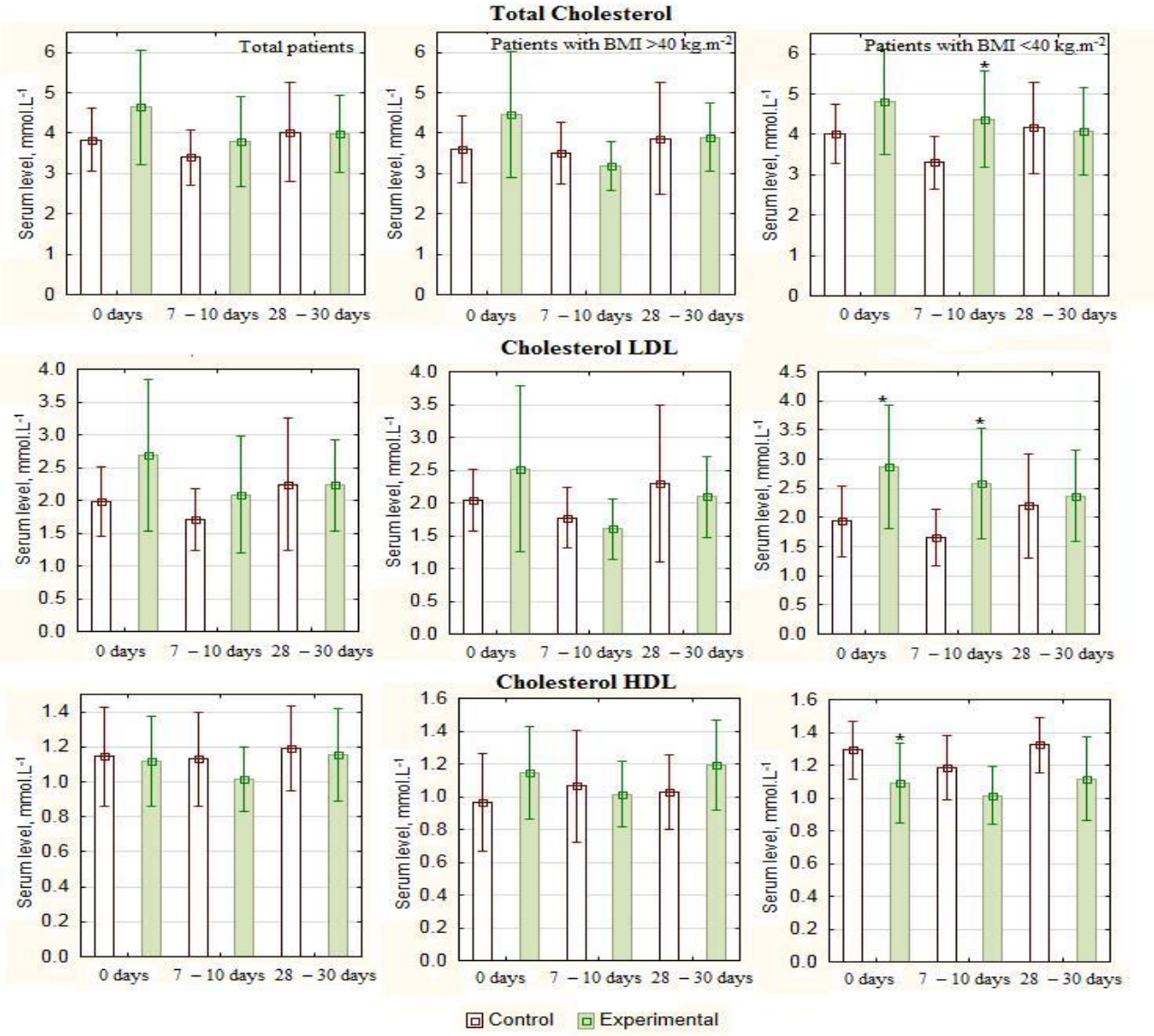

Figure 1 Cholesterol distribution in the serum of patients during the trial. Note: * - significant difference between the control and experimental groups (p <0.05)

In the control group, a statistically significant cholesterol LDL reduction by 13.8% was noticed after 7 – 10 days, mainly due to its reduction in the serum of patients with a BMI below 40 kg.m^{-2} by 14.1%. LDL cholesterol level decline was also marked in the serum of experimental patients and was equal to 22.4% after 7 – 10 days, mainly due to its reduction in the serum of patients with a higher BMI of 40 kg.m^{-2} by 36.4%. The cholesterol LDL level returned to its original level in both groups after 28 – 30 days of dieting. The same tendency was noticed concerning the cholesterol HDL level.

There were no significantly reliable changes to triglyceride levels and serum atherogenic index in both groups. However, after 7–10 days after the diets, a decrease in triglyceride levels in the control group amounted to only 3.9% and returned to its original level after 28 – 30 days, while the reduction in experimental patients was equal to 12.0% and continued to reduce until 28 – 30 days to 17.4%. The same tendency was noticed concerning serum atherogenic index (AI). Also, 7 – 10 days after the diets, the decrease in AI in the control group amounted to 16.4% and returned to its original level after 28 – 30 days, while the reduction was equal to 13.7% in experimental patients and continued to reduce until 28 – 30 days to 20.4%.

Cholesterol levels on days 0, 7 – 10 and 28 – 30 days were compared between the control and experimental groups according to the Mann-Whitney U test. There were no significantly reliable changes in cholesterol levels in the two groups for all patients and patients with a BMI higher 40 kg.m^{-2}. Cholesterol LDL on day 0 in the serum of experimental patients with a BMI lower than 40 kg.m^{-2} was higher than in the control group by 48.3% (p <0.05), while cholesterol HDL was lower by 15.7%; therefore, there was no difference between total cholesterol levels.

After 7 – 10 days, the cholesterol LDL in the serum of experimental patients with a BMI below 40 kg.m^{-2} was still higher than in the control group by 55.4% (p <0.05), but returned to the original level in both groups after 28 – 30 days of dieting.

With such heterogeneity of groups, it is advisable to consider changes in the lipid profile in dynamics and compare changes in parameters between groups. Dynamics were evaluated between 0 day and 7 – 8 days and 0 day and 28 – 30 days and are presented in Table 2 in percentiles ($P_{25\backslash75}$); statistical differences were calculated according to Mann-Whitney U tests.

There were no significantly reliable changes in Δ total cholesterol after 7 – 10 days of diet consumption, but after 28 – 30 days in the serum of experimental patients it was significantly lower than in the control, mainly due to LDL cholesterol reduction especially in patients with a BMI below 40 $kg.m^{-2}$. The same tendency was noticed concerning Δ triglycerides. There was still no significantly reliable changes in the Δ serum atherogenic index, despite its remarkable reduction in the experimental group by 13.7% and 20.4% at 7 – 10 and 28 – 30 days, respectively (Table 1). Nevertheless, a positive effect of MP on the activity (**Chernukha et al., 2016; Chernukha et al., 2018; Kotenkova and Chernukha, 2019**).

On the other hand, numerous authors also considered animal proteins as abundant sources of functional peptides, including those which demonstrated a lipid-lowering effect **(Bauchart et al., 2006; Ahhmed and Muguruma, 2010; Toldrá et al., 2012; Udenigwe and Howard, 2013; Lafarga and Hayes, 2014; Cicero, Fogacci and Colletti, 2017)**. **Nakade et al. (2009)** revealed that cattle heart protein hydrolysate could suppress cholesterol absorption in Caco-2 cells.

It is known that aorta tissue is enriched with collagen and elastin, while collagen-derived peptides have been better studied and are characterised by their lipid-lowering effect **(Koyama and Kusubata, 2013; Hongdong and Bo, 2017; Tometsuka et al., 2017; Tomosugi et al., 2017; Yazaki et al., 2017; Arrutia et al., 2017; Wahart et al., 2019)**. In this regard, aorta tissue is a good source of

Table 2 Dynamics of serum lipid profile changes in patients during the trial.

Days	Control group			Experimental group		
	Total	BMI >40 $kg.m^{-2}$	BMI <40 $kg.m^{-2}$	Total	BMI >40 $kg.m^{-2}$	BMI <40 $kg.m^{-2}$
Δ total Cholesterol, $P_{25/75}$						
7 – 10	-0.024/1.314	-0.380/1.211	-0.008/1.502	0.349/0.784	0.372/2.139	0.325/0.652
28 – 30	-0.597/0.368	-0.556/0.117	-0.608/0.953	-0.218/1.631*	-0.406/1.564	-0.045/1.698
Δ Cholesterol LDL, $P_{25/75}$						
7 – 10	-0.094/0.756	-0.163/0.753	-0.025/1.010	0.244/0.687	0.279/1.682	0.110/0.627
28 – 30	-0.465/0.034	-0.325/0.050	-0.642/0.017	-0.142/1.144*	-0.399/1.274	-0.114/0.977*
Δ Cholesterol HDL, $P_{25/75}$						
7 – 10	-0.036/0.177	-0.038/0.153	0.045/0.186	-0.014/0.206	0.002/0.223	-0.028/0.141
28 – 30	-0.186/0.077	-0.173/-0.019	-0.198/0.082	-0.148/0.047	-0.155/0.074	-0.137/0.020
Δ Triglycerides, $P_{25/75}$						
7 – 10	-0.083/0.218	-0.226/0.246	-0.071/0.216	-0.273/0.421	-0.215/0.395	-0.303/0.446
28 – 30	-0.174/0.125	-0.081/0.081	-0.296/0.168	-0.148/0.794*	-0.183/0.862	-0.051/0.606*
Δ Serum atherogenic index, $P_{25/75}$						
7 – 10	-0.155/0.730	-0.207/0.944	-0.103/0.713	-0.023/0.822	-0.017/1.493	-0.158/0.605
28 – 30	-0.537/0.703	0.101/0.475	-0.693/0.822	-0.021/1.489	-0.040/1.642	-0.002/1.444

Note: * -significant differences between the control and experimental groups (p <0.05)

serum lipid profile of patients during the trial is noticeable.

In a previous study it was shown that meat products characterised by milder hypolipidaemic action compared with native raw material or isolated active fractions; a slight effect was observed after 28 days of consumption by hyperlipidemic rats, while a significant effect was only seen after 42 days of consumption **(Kotenkova and Chernukha, 2019)**.

The pronounced effect of meat product could be linked to the unique proteome and peptidome of the heart and aorta tissues based on organ-specific gene expression and the presence of tissue-specific substances **(Fagerberg et al., 2014; Breschi et al., 2016; Guschanski, Warnefors and Kaessmann, 2017; Sonawane et al., 2017; Barbeira et al., 2018)**. Previously, we also observed that sterilisation led to the decomposition of most parts of the target tissue-specific proteins and peptides into active peptides with apparently similar biological actions or retained residual glyproline peptides with hypolipidaemic action **(Lyapina et al., 2015; Shabalina Lyapina et al., 2015; Myasoedov et al., 2016)**. Presumably, active peptides could be generated both during meat product processing and digestion processes.

CONCLUSION

The positive effect of developed meat products on the serum lipid profile of patients during the trial was mild but noticeable. Presumably, it is more preferable to use this as a component in a diet therapy for hyperlipidaemic humans for 28 – 30 days. Earlier, the same effect was revealed on hyperlipidaemic rodents, which confirms the lipid decreasing ability effect of innovative products and can be recommended as part of a diet to fight CVD. The pronounced effect of the product could be linked to the unique proteome and peptidome of the heart and aorta tissues based on organ-specific gene expression and the presence of tissue-specific substances.

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UTILISATION OF QUINOA FOR DEVELOPMENT OF FERMENTED BEVERAGES

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ABSTRACT

Lactic acid fermentation of pseudocereals represents a useful tool for the preparation of value-added beverages with beneficial properties to consumers. The aim of this work was the development of a novel quinoa-based beverage fermented with commercially available probiotic culture including *Bifidobacterium sp., Lactobacillus acidophilus,* and *Streptococcus thermophilus*. The results concluded that fermentation of quinoa beverages significantly increased proteins and total phenolic content and antioxidation activity in the final products (by 36.84%, 26.67%, and 14.74%, respectively). In general, the overall acceptability of unfermented quinoa beverages was low (less than 46%), but the fermentation process slightly increased their acceptability (by 9.43%). A significant improvement of acceptability was observed, when the raspberry syrup was supplemented into the fermented beverages (by 90.98% compared to the no supplemented samples). Viability of fermenting microorganisms, pH, total acidity, and organic acid content were determined during the storage of beverages for 21 days at 5 °C. It was found that prepared quinoa beverages had a good probiotic potential (>6 CFU.mL^{-1} of lactic acid bacteria cocci). Furthermore, this study also showed that the quinoa represents a suitable raw material for formulation novel gluten and dairy-free fermented beverages with increased content of nutritionally important compounds.

Keywords: quinoa; fermentation; lactic acid bacteria; beverages; sensory acceptance

INTRODUCTION

Recently, there is a growing demand for new types of cereal products with a higher nutritional value, which can offer health benefits to consumers due to the content of biologically active substances **(Dallagnol et al., 2013)**. In this respect, considerable attention is focused on the use of pseudocereals, mainly quinoa (*Chenopodium quinoa*) **(Dallagnol et al., 2013; Bhargava, Shukla and Ohri, 2006)**. Quinoa is regarded one of the best vegetal protein sources (12 – 23%), as its protein levels are similar to those found in milk and higher than in true cereals such as wheat, rice and maize **(Gordillo–Bastidas et al., 2016; Kaur and Tanwar, 2016; Nisar et al., 2017; Zannini et al., 2018).** Moreover, quinoa contains a significant amount of starch (52 – 60%) with low amylose content (7 – 11%) **(Ahmed, Thomas and Arfat, 2019)** and the amount of dietary fibre in the quinoa is higher than in the other grains (9 – 16%) **(Gordillo–Bastidas et al., 2016)**. In addition, quinoa is a rich source of bioactive compounds like antioxidants, polyphenols, flavonoids, minerals (magnesium, zinc, iron, potassium, phosphorus) and vitamins (E, B group and C) **(Kaur and Tanwar, 2016; Tang and Tsao, 2017; Ahmed, Thomas and Arfat, 2019)**.

Due to the above mentioned nutritional benefits, the quinoa seeds have recently been incorporated into the functional food products **(Lorruso et al., 2018; Ujiroghene et al. 2019)**. Several authors investigated the potential of quinoa for the production of beverages with using various methods of treatment such as soaking, germinating, cooking, malting **(Pineli et al., 2015; Kaur and Tanwar, 2016; Urquizo et al., 2017; Zannini et al., 2018)**. On the other hand, only a few research works are focused to the production of probiotic quinoa-based fermented beverages **(Bianchi et al., 2014; Lorusso et al., 2018)**. Currently, the study of **Lorusso et al. (2018)** was oriented to assessment the suitability of quinoa for making yogurt-like beverages fermented by using probiotic lactic acid bacteria strains (*Lactobacillus rhamnosus* SP1) and **Bianchi et al. (2014)** developed a potentially synbiotic beverage fermented with *Lactobacillus casei* LC-1 based on aqueous extracts of soy and quinoa with added fructooligosaccharides. More recently **Ujiroghene et al. (2019)** reported that quinoa-based fermented beverages can be applied for diabetes mellitus treatment due to their ability to inhibit α-amylase activity and reduction or prevence hyperglycemic conditions associated with increased levels of sugar glucose in the blood.

Scientific hypothesis

In recent decades, there is an increasing interest of consumers related to the consumption of value-added beverages. In response to consumer demands, this study aimed to evaluate if quinoa could be used for the production of fermented probiotic beverages and could increase its biological value, protein, phenolic content, and antioxidant activity. The sensory acceptability of fermented beverages and their stability during storage was also determined.

MATERIAL AND METHODOLOGY

Raw materials

Quinoa seeds and raspberry syrup purchased from the local Slovak market were used in this study. Before using seeds for the preparation of fermented beverages, the seeds were desaponificated according to the method described by **Urquizo et al. (2017)**. Dried commercial probiotic culture of Lactoflora (Milcom a.s., Prague, Czech Republic) including *Bifidobacterium sp., Lactobacillus acidophilus,* and *Streptococcus thermophilus* was used for the preparation of fermented quinoa beverages.

Preparation of fermented quinoa beverages

Fermented beverages were prepared according to the modified procedure previously documented by **Urquizo et al. (2017)**. Desaponificated quinoa seeds were dried at 60 °C for 8 hours and milled to flour. In the next step, the quinoa flour was mixed with tap water at a concentration of 5% (w/v), afterward, the mixture was gelatinized at 95 °C for 10 min and cooled to 20 °C. The dried commercial probiotic culture was used for direct inoculation of beverage after dilution (initial total lactic acid bacteria count: 10_8 CFU.mL^{-1}). Samples were fermented at 37 °C for 6 hours. After fermentation, the beverages were stored in closed glass containers for 21 days at 5 °C.

Proximate analyses

Proximate analyses of beverages included determination of dry matter (AACC method 44-19.01), proteins by the method of Kjeldahl using a factor of 6.25 (AACC method 46-13.01), crude fat (AACC method 30-25.01) and ash (AACC method 08-01.01) **(AACC, 2000)**. Total dietary fibre (TDF) content was determined by enzymatic-gravimetric method 985.29 **(AOAC, 2003)**. Saponin content in seeds before and after desaponification was measured according to the method described by **Koziol (1991)**. pH of beverages was determined by using a pH-meter (Inolab WTW, Weilheim, Germany). Total acidity was measured by the visual titration method with a standard solution of NaOH (0.1 mol.L^{-1}) and using phenolphthalein as the indicator. Lactic, acetic, and citric acids were determined using the method of capillary isotachophoresis **(Kohajdová Karovičová and Greifová, 2006; Magala et al., 2015)**. Isotachophoretic measurements were realised using an isotachophoretic analyser and the ZKI01 columns connection technique (Villa Labeco, Spišská Nová Ves, Slovak Republic), equipped with a conductivity detector and two-line recorder TZ 4200 (Laboratory instrument, Prague, Czech Republic). The total phenolic content (TPC) was determined using the Folin-Ciocalteu assay **(Park et al., 2017)**. The analysis of TPC was realised by comparison to a calibration curve constructed by gallic acid. Amount of TPC was expressed as milligrams of gallic acid equivalents (GAE) per 100 g of quinoa sample. Antioxidant activity was monitored by DPPH (1,1-Diphenyl-2-picryl-hydrazyl) assay according to the method described by **Kaur and Tanwar (2016)**.

Microbiological analyses

Presumptive counts of lactic acid bacteria cocci and presumptive counts of lactic acid bacteria rods were determined after dilution and cultivation on M17 and MRS agar plates respectively according to STN ISO method 15214 **(STN ISO 15214, 2002)**.

Technological properties

The viscosity of beverages was measured using rotation viscosimeter (Haake VT 550, Haake Mess – Technic, Germany) according to the method reported by **Magala et al. (2015)**. Water holding capacity (WHC) of beverages was determined according to the method previously described by **Zannini et al. (2018)**.

Sensory evaluation

Sensory characteristics of beverages were evaluated by panel assessors (11 – member panel) using a 5 – points hedonic scale (1 – dislike extremely; 2 – dislike slightly; 3 – either like nor dislike; 4 – like slightly; 5 – like extremely) **(Graham, Agbenorhevi and Kpodo, 2017)**. The assessors evaluated overall appearance, taste, odour, colour, and consistency of fermented products. The overall acceptability of beverages was evaluated using 100 mm graphical unstructured line segments with specified end-points **(Kohajdová, Karovičová and Greifová, 2006)**.

In the next step, raspberry syrup sweetened with fructose (selection was performed concerning persons treated to diabetes as potential consumers) was used for increasing sensory acceptability of fermented beverages. After fermentation, the appropriate part of beverages were mixed with raspberry syrup (10%, v/v) and evaluated. The level of this ingredient was chosen on the basis of the previously performed preliminary trial.

Statistical analysis

All analyses were carried out using three independent determinations and expressed as the mean value ± standard deviation. Statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) with XLSTAT for MS Excel Addinsoft SARL, Paris, France). Differences among means were analyzed by Student's *t* - test. The significance level (p) was set at 0.05.

RESULTS AND DISCUSSION

The fermentation of cereals improves the nutritional properties and sensory characteristics of final products and has a positive impact on human health **(Rollán, Gerez and LeBlanc, 2019)**. Nowadays, many scientific studies are focused on the preparation of new types of cereal-based fermented beverages with probiotic and functional properties **(Rathore et al., 2012; Nionelli et al. 2014;**

Ghosh et al. 2015; Magala et al., 2015). Therefore, considering the nutritional and health benefits of quinoa, in this study, the fermented quinoa-based beverages with probiotic potential were developed.

Saponins are bitter compounds that are naturally present in quinoa seeds in the range from 0.1 to 5.0% **(Pytel et al., 2018)**. The content of saponins in the quinoa seeds used in this study was 0.30 ±0.02% (results are not shown). For remove of saponins, washing or water maceration is recommended **(Pineli et al., 2015)**. Washing with tap water was used for removing of saponins in this study. After desaponification, the amount of saponins in quinoa seeds was present in the non-detectable level. Previously **Pytel et al. (2018)** demonstrated a reduction number of lactic acid bacteria during fermentation of yogurts with 3 and 5% content of quinoa flour. Moreover, it was reported that saponins present in the quinoa seeds also cause a negative effect on protein solubility **(Pineli et al., 2015)**. For these reasons, the removal of saponins from the quinoa seeds representing an important step before the production of lactic acid fermented quinoa-based beverages. Proximate composition of quinoa-based beverages before and after 6 h fermentation is presented in Table 1.

During the fermentation process, the pH decreased and total acidity increased significantly in the beverages. This effect was previously described by **Magala et al. (2015)** in rice fermented beverages and **Urquizo et al. (2017)** in the quinoa fermented beverages prepared from various quinoa varieties. A rapid decrease of the pH at the start of fermentation is important to obtain the final product of high quality **(Kohajdová and Karovičová, 2008)**, as well as the rapid increase of total acidity minimizes the growth of undesirable and pathogenic bacteria **(Kohajdová Karovičová and Greifová, 2006)**.

During the lactic acid fermentation, different organic acids (mainly lactic acid) are produced due to the degradation of some components present in the raw material **(Kohajdová, Karovičová and Greifová, 2006; Magala, Kohajdová and Karovičová, 2013; Cho et al., 2015)**. Determination of organic acid profile represents an important tool for monitoring the metabolic activity of fermentation microorganisms **(Cho et al., 2015)**. In this study, the significant increasing of lactic and acetic acids concentration was observed in the fermented beverages in comparison to unfermented samples. Furthermore, slightly decreasing in citric acid content was recorded. This can be attributed to its usage as a substrate in secondary reactions during fermentation process **(Magala, Kohajdová and Karovičová, 2013)**.

Moreover, it was found that, the lactic acid fermentation no significantly influenced dry matter, ash and crude fat content in the quinoa-based beverages. On the other hand, however, after fermentation a significant increasing in protein content was observed in the beverages (increasing about 36.84%). **Tangyu et al. (2019)** reported that fermentation can increase protein content by the growth of the fermenting microorganisms and by improving protein solubility. According to **Simwaka et al. (2017)** the increase in protein content after fermentation has been attributed to the increase in nitrogen content released when microorganisms utilize carbohydrates for energy.

From the results also concluded that the fermentation not significantly influenced the content of TDF in the samples of beverages. Similar trend was also observed by **Marko et al. (2014)** in different fermented cereal matrices. On the other hand, **Jorgensen et al. (2010)** reported that the fermentation of cereals substrates can concluded in reduction of TDF content as a consequence the enzymatic degradation of fibre.

Table 1 Proximate composition, microbial counts and technological properties of quinoa-based beverages.

Fermentation (h)	0	6
Proximate composition		
pH	6.89 ±0.15	4.19 ±0.05*
Total acidity (mmol.L-1)	1.01 ±0.07	5.50 ±0.13*
Lactic acid (g.L-1)	0.25 ±0.01	0.46 ±0.02*
Acetic acid (g.L-1)	0.15 ±0.01	0.26 ±0.01*
Citric acid (g.L-1)	0.22 ±0.01	0.17 ±0.00
Dry matter (%)	5.63 ±0.04	5.83 ±0.02
Ash content (%)	0.04 ±0.00	0.05 ±0.00
Proteins (%)	0.57 ±0.04	0.78 ±0.02*
Crude fat (%)	0.11 ±0.01	0.09 ±0.01
TDF (%)	0.43 ±0.03	0.42 ±0.02
TPC (mg GAE per 100 g of sample)	142.37 ±1.27	180.33 ±1.25*
Antioxidant activity (%)	60.31 ±2.08	69.2 ±1.98*
Microbiological analyses		
Presumptive count of lactic acid bacteria cocci (log CFU.mL^{-1})	6.13 ±0.05	7.21 ±0.08*
Presumptive count of lactic acid bacteria rods (log CFU.mL^{-1})	4.47 ±0.13	6.12 ±0.13*
Technological properties		
Dynamic viscosity (mPa.s)	176.88 ±7.33	236.78 ±6.26*
WHC (%)	74.68 ±2.50	73.90 ±3.32

Note: 0 – quinoa beverages before fermentation, 6 – quinoa beverages after 6h fermentation, TDF – total dietary fiber, TPC – total phenolic contents, GAE – gallic acid equivalents, WHC – water holding capacity, * denotes that means within a line differ significantly (p <0.05).

Table 2 Sensory characteristics of quinoa based beverages.

Fermentation (h)	0	6	6^{x}
	Sensory characteristics		
Overall appearance	2.50 ±0.02	3.31 ±0.04*	4.18 ±0.12*
Taste	2.15 ±0.04	2.77 ±0.06*	4.00 ±0.21*
Odour	2.08 ±0.07	2.46 ±0.09*	4.54 ±0.18*
Colour	3.00 ±0.12	2.88 ±0.10	3.54 ±0.04
Consistency	3.08 ±0.02	2.98 ±0.09	3.10 ±0.09
Overall acceptability (%)	41.67 ±1.03	45.60 ±1.05	79.58 ±2.84*

Note: 0 – quinoa beverages before fermentation, 6 – quinoa beverages after 6h fermentation, 6^{x} – quinoa beverages after 6h fermentation with addition of raspberry syrup, * denotes that means within a line differ significantly (p <0.05).

0 **6** **6^{x}**

Figure 1 Photodocumentation of quinoa-based beverages.
Note: 0 – quinoa beverages before fermentation, 6 – quinoa beverages after 6h fermentation, 6^{x} – quinoa beverages after 6h fermentation with addition of raspberry syrup.

Table 3 Metabolic activity during storage of quinoa based fermented beverages.

Storage (days)	0	7	14	21
pH	4.19 ±0.05	3.99 ±0.04	3.95 ±0.07	3.91 ±0.11
Total acidity (mmol.L^{-1})	5.15 ±0.13	8.00 ±0.21*	10.01 ±0.32*	10.50 ±0.59*
Lactic acid (g.L^{-1})	0.46 ±0.02	0.43 ±0.08	0.48 ±0.01*	0,49 ±0.03*
Acetic acid (g.L^{-1})	0.21 ±0.01	0.19 ±0.03	0.18 ±0.02	0.18 ±0.04
Citric acid (g.L^{-1})	0.17 ±0.00	0.11 ±0.02*	0.03 ±0.00*	ND
Presumptive count of lactic acid bacteria cocci (log CFU.mL^{-1})	7.21 ±0.08	7.13 ±0.16	7.01 ±0.02*	6.12 ±0.08*
Presumptive count of lactic acid bacteria rods (log CFU.mL^{-1})	6.12 ±0.13	5.79 ±0.20*	5.57 ±0.14*	4.94 ±0.01*

Note: * denotes that means within a line differ significantly (p <0.05), ND – non detectable.

Since TDF represent an important functional compound with beneficial health effects, the incorporation of fibre rich raw materials obtained from plant processing by products into the non-dairy beverages can be a good strategy for increasing intake of TDF. In this respect, it seems to be a good alternative application of apple pomace as a fibre rich (more than 50% of TDF) by-product obtained from the juice processing industry that was usefully incorporated to cereal-based products in our previous study **(Kohajdová et al., 2014)**. Moreover, this by product can improve sensory acceptance of non-dairy fermented beverages as a flavouring ingredient due to pleasant fruity odour and taste **(Sudha, Baskaran and Leelavathi, 2007)**.

The phenolic compounds are important for human health and nutrition mainly due to their antioxidant activities **(Hole et al., 2012; Tang and Tsao, 2017)**. As shown in Table 1, the significant increasing of TPC was observed in the quinoa-based fermented beverages. A similar effect was also reported by **Hole et al. (2012)** and **Lorusso et al. (2018)** for the barley, oat, and quinoa substrates processed by lactic acid fermentation. Previously **Bustos et al. (2017)** and **Lorusso et al. (2018)** reported that, during the fermentation process the esterase activity of lactic acid bacteria and endogenous enzymes of cereal substrates can hydrolyse the complex phenolic compounds and their glycosylated forms into free phenolic acids.

Several studies documented that the quinoa seeds are an excellent source of antioxidants and that, the antioxidant

activity is in a good correlation with the content of TPC **(Kaur and Tanwar, 2016; Tang and Tsao, 2017)**. This fact was also confirmed in this study when the increased TPC content in the fermented beverages corresponded to a proportional increase of the antioxidant activity of fermented products.

After 6 h fermentation, the content of lactic acid bacteria cocci and rods reached values 7.21 log CFU.mL^{-1} and 6.12 log CFU.mL^{-1} respectively. The population of lactic acid bacteria in the fermented beverages was increased significantly by 1.08 (cocci) and 1.65 (rods) log orders. This indicates that quinoa is a suitable substrate for lactic acid fermentation.

Consistency represents an important parameter for the development of new functional fermented non-dairy beverages **(Magala et al., 2015)**. The viscosity of quinoa-based beverages significantly increased during the fermentation process. This agreed with **Bianchi et al. (2014)** and **Lorusso et al. (2018)** that also observed increased viscosity in the fermented beverages produced from aqueous extract of quinoa and yogurt-like quinoa beverages. **Ndife et al. (2019)** reported that the increase in the viscosity of cereal beverages after fermentation could be due to an increase in biomass density of the microorganisms.

No significant differences were found in the WHC of beverages before and after fermentation. This indicates that the WHC of beverages remaining stable during the fermentation process.

Sensory characteristics of quinoa-based beverages are shown in Table 2 and photodocumentation of quinoa-based beverages is presented in Figure 1.

Väkeväinen et al. (2020) newly documented that the saponins present in the quinoa seeds decrease the acceptance of quinoa-based products. Following this recommendation and due to the other fact presented in the above part of this article, the quinoa seeds were desaponificated before the use in beverages manufacture. The results concluded that the overall acceptance of nonfermented beverages was lower than 42%. Lactic acid fermentation significantly enhanced overall appearance, taste and odour, but no significantly influenced the overall acceptability of beverages. Fermented beverages were characterised with creamy light colour and sour odour and taste. Due to the low sensory acceptability of beverages, the raspberry syrup was applied as a supplement to improve their acceptability. This ingredient was sweetened with fructose, a sweetener with a low glycemic index that is usually consumed by persons treated to diabetes. The addition of this supplement significantly improved the overall acceptability of fermented beverages. A similar trend was observed by **Urquizo et al. (2017)** after the addition of bilberries and chocolate flavouring to lactic acid fermented quinoa beverages.

The shelf life of fermented beverages was also monitored for 21 days of storage at 5 °C (Table 3). During the fermentation, the pH of beverages slightly decreased. Moreover, the significant increasing of total acidity was recorded. A similar trend was previously described by **Bianchi et al. (2014)** after 28 days of storage of fermented quinoa and soy beverages at 5 °C. Furthermore, it was found that no significant differences were recorded in the content of lactic and acetic acids. The results also concluded that during storage, the citric acid was degraded as the consequence of the metabolic activity of fermenting microflora.

The presumptive count of lactic acid bacteria was also determined in this study. It was observed that lactic acid that presented bacteria cocci and rods showed a high rate of survival percentage during the storage of beverages (84.88 and 80.72% respectively). The viability of bacteria is an important characteristic of the use of probiotics in beverages, once they should survive during the shelf life, with minimal desirable value 6 log CFU.mL^{-1} **(Georgieva et al., 2009; Gallina et al., 2019)**. This value corresponds to ingestion an 8 log CFU per serving portion of 100 mL **(Pereira and Rodrigues, 2012)**. It was found that lactic acid bacteria cocci (*Streptococcus thermophilus*) were present in the beverages at the level above this requirement. On the other hand, the population of lactic acid bacteria rods (*Bifidobacterium* sp*., Lactobacillus acidophilus*) reached at the end of storage only counts 4.94 log CFU.mL^{-1}. **Kurman and Rasic (1991)** and **Samona and Robinson (1994)** previously suggested that the minimum level for probiotic microorganisms in fermented milk to produce therapeutic benefits should be a value above 5 log CFU.mL^{-1}. After 14 days of storage the population of lactic acid bacteria rods remained above this value. From the obtained results can be indicated that experimental beverages had a probiotic character.

CONCLUSION

The potential of quinoa as a raw material for the production of lactic acid fermented beverages was presented in this study. It was shown that the fermentation process significantly increased proteins and total phenolic content and antioxidation activity in the products. Sensory evaluation showed that the overall acceptability of quinoa-based fermented was lower than 46%. The addition of flavouring agent (raspberry syrup) significantly improved acceptability of beverages (79.58%). Regarding these finding, the application of flavourants could be a useful strategy for enhancing consumers' acceptance of these types of beverages. Moreover, further studies are needed to select suitable flavouring agents according to the preferences of potential consumers. The functionality of commercial probiotic culture was demonstrated by high viable bacterial counts in the fermented beverages (7.21 log CFU.mL^{-1} of lactic acid bacteria cocci and 6.12 log CFU.mL^{-1} of lactic acid bacteria rods) and during the storage (above 6 CFU.mL^{-1} of lactic acid bacteria cocci).

In conclusion, the quinoa is naturally gluten-free raw material and thus, quinoa-based fermented beverages represent a suitable alternative for people having celiac disease. Moreover, quinoa has a low glycemic index and in combination with appropriate ingredients, these beverages can be incorporated into the diabetic diet.

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FRUIT RED COLORANTS IMPACT ON THE ANTIRADICAL ACTIVITY DETERMINED BY DPPH METHOD

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ABSTRACT

This work is focused on the determination of antiradical activity (ARA) by the method of free radical scavenging of 2,2-Diphenyl-1-picrylhydrazyl (DPPH). Since the DPPH solution is intense purple-coloured and absorbs at a wavelength of 517 nm, similar to the anthocyanin colorants of fruits, the modification of the method examined the effect of the colour of the sample extracts on the result of the ARA determination. Statistical evaluation of the results of the analyses using Youden's graphing method revealed that the two compared method adjustments gave consistent results over the observed range of antiradical activity. It also showed that there was no statistically significant difference between the mean ARA values obtained by the two treatments. Investigation of the effect of the evaluated fruit components revealed a strong correlation between the content of ascorbic acid and ARA in samples containing no anthocyanin colorants. For the fruit samples studied, ARA values showed a strong correlation with polyphenol content.

Keywords: DPPH method; antiradical activity; dependence; ascorbic acid; polyphenols

INTRODUCTION

The antioxidant capacity is often measured by a method based on decolorizing stable radicals of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) with various antioxidants. This method is simple, efficient, and relatively cheap. DPPH is commercially available, stable, and poorly sensitive to degradation, which will significantly affect its use. The DPPH colour is intense purple and shows a strong absorption band in the 515-520 nm range. The original method of DPPH analysis was developed by **Marsen Blois (1958)**, which later underwent various modifications **(Brand-Willams, Cuvelier, and Berset, 1995; Chen, Bertin, and Froldi, 2013)** and are also widely used. The DPPH method is a mixed HAT (Hydrogen Atom Transfer) and SET (Single Electron Transfer) method, since, in the presence of an antioxidant molecule, DPPH can receive one electron or even one proton from the antioxidant molecule. This turns it into a more stable reduced form, which also changes its intense purple colour to faint yellow. The antiradical activity of the sample (ARA) can be quantified by spectrophotometric measurement of this conversion.

Measurement results are often expressed as "EC50 value", ("efficient concentration" or EC50 value), which represents the substrate concentration to achieve a 50% reduction in the original DPPH concentration. This parameter was introduced by **Brand-Willams et al. (1995)** and subsequently applied by a few adjustments to several groups of workers to present their results **(Kedare and Singh, 2011)**. This parameter depends on the initial DPPH concentration as well as the chosen reaction time chosen by the authors over a relatively wide time span. This makes the comparability of the results more difficult but gives a good idea of the ARA value of the samples analysed.

Although considered to be simple and effective, this method is characterized by various limitations that make measurement difficult. Many studies have pointed out that there is no linear dependence between antioxidant concentration and DPPH radical scavenging activity. Thus, the EC50 expression can be problematic in many cases **(Mishra, Ojha, and Chaudhury, 2012; Carmona-Jiménez et al., 2014; Sánchez-Moreno, Larrauri and Saura-Calixto, 1998)**.

Some authors recommend measuring the result of a blank sample **(Chang et al., 2001; Lachman et al., 2006; Jakobek et al., 2008; Vollmannová et al., 2013; Žiarovská et al., 2014)**, others recommend drawing the results to the absorbance value at time T_0 for each sample separately **(Dawidowicz, Wianowska, and Olszowy, 2012)**, whereby the possible interference of other colorants in the extract (e.g. carotenoids and anthocyanins) at the wavelength used of 517 nm would be eliminated **(Apak et al., 2007)**.

The antioxidant activity itself is influenced by the presence of many compounds present in natural materials.

First of all, it is a question of polyphenols and vitamin C **(Jakobek and Seruga 2012; Hegedűs et al., 2015)**. The influence of content substances on antioxidant activity was also investigated by **Ivanišová et al. (2010)** and showed a very high correlation between DPPH content and polyphenol content and vitamin C content.

Scientific hypothesis

Based on the fact that samples of fruit extracts with a high content of anthocyanins strongly absorb wavelength light as well as DPPH solution, this may lead to differences in results when analysing intensively coloured extracts. The main objective of the work was based on the assumption that by modifying the method it is possible to correct this effect.

As the ARA value is influenced by different compounds, we assumed a dependence between the determined ARA value and some components by analysing a wide range of fruits.

MATERIAL AND METHODOLOGY

Chemicals and Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and L-ascorbic acid CertiPUR were purchased from Merck, Darmstadt, Germany, Methanol from Fisher Scientific UK, Loughborough, UK, Gallic acid from MP Biomedicals, LLC, France and Folin-Ciocalteu reagent from VWR International S.A.S., France. All other reagents were of analytical reagent grade. Deionized water was used to prepare all solutions.

Instrumentation

Spectrophotometer Jenway 6301, Bibby Scientific Ltd., UK was used for absorption measurement. WATERS HPLC system with Waters 2489 UV/VIS Detector was used for ascorbic acid determination.

Samples and Sample Preparation

For the testing method purpose, we analysed 19 fruits from several growers from the districts of Komárno and Nové Zámky (elevation 110 – 150 m a.s.l.) from southern Slovakia. The assortment of fruits was supplemented with samples of southern fruits: bananas, lemons, and oranges, which were purchased from the retail network. These were the following fruit species: apricot (*Prunus armeniaca* L.), raspberry (*Rubus idaeus* L.), sour cherry *(Prunus cerasus* L.), blackcurrant (*Ribes nigrum* L.), redcurrant (*Ribes rubrum* L.), black mulberry (*Morus nigra* L.), josta (*Ribes* × *nidigrolaria* Rud. Bauer et A. Bauer), greengage yellow and red (*Prunus domestica* L. *subsp. italica* (Borkh.) Gams ex Hegi), gooseberry green (*Ribes uva-crispa* L.), gooseberry pink (*Ribes uva-crispa* L.), white mulberry (*Morus alba* L.), blueberry (*Vaccinium myrtillus* L.), cherry plum Nigra (*Prunus cerasifera* Ehrh. *subsp. pissartii* (Carriere) C. K. Scneid. cv. *'Nigra'*), Sea buckthorn (*Hippophae rhamnoides* L.), common fig (*Ficus carica* L.), summer apple (*Malus domestica* Borkh.), white and red grapes (*Vitis vinifera* L.), banana (*Musa x paradisiaca* L.), orange (*Citrus sinensis* (L.) Pers.), lemon (*Citrus limon* (L.) Burm.).

For the determination of ARA and polyphenols, methanol extracts were obtained from average samples of individual fruit species by extraction of 10 g of the sample in 70% methanol, which was used for spectrophotometric measurement.

Methods of determination

ARA was determined by DPPH and expressed as % inhibition of DPPH radicals per g of sample **(Hegedűs et al., 2019a)**. Two modifications of the method were used:

- the absorbance results of the samples 30 min after the addition of DPPH were related to the initial absorbance of each sample separately,
- the absorbance results of the samples 30 min after the addition of DPPH were related to the absorbance of the blank.

The absorbance of the samples was measured at a wavelength of 517 nm.

The anti-radical activity was calculated as the percentage of DPPH discoloration per 1 g sample using the following formula:

$$\% \text{ ARA} = (1 - A_{t30} / A_{t0}) * 100 / n * V_2 / V_1) \quad (1)$$

Where: A_{t30} – absorbance of the sample after 30 min; n – weigh of the sample in g; V_1 – the pipetted volume of the sample (0.1 to 2.0 mL); V_2 – supplemented volume of the extract by methanol (according to the stated method always 2.0 mL); A_{t0} – the initial sample absorbance value.

Total phenolic contents were determined using the Folin-Ciocaltu method, using Gallic acid as standard **(Sánchez-Rangel et al., 2013)**. From the prepared sample extracts, 0.1 to 1.0 ml was pipetted and made up to 1.0 mL with deionized water. After standing for 5 min, 5.0 mL of Folin-Ciocalteu reagent, and 4.0 mL of 7.5% sodium carbonate solution were added. After 1 hour, the absorbance of the examined solutions is measured at 765 nm.

The determination of L-ascorbic acid was performed by the HPLC method, using sample extract in 2% oxalic acid medium **(Hegedűs et al., 2019b)**.

Statistical analysis

Statistical evaluation of the two averages was performed by paired t-test at the significance level $\alpha = 0.05$. The hypothesis was tested:

H_0: $\bar{x}_A = \bar{x}_B$ against the alternative

H_1: $\bar{x}_A \neq \bar{x}_B$, where $\bar{x}$ is the average value.

The evaluation of two modifications of the method in the obtained range of ARA values was made using Youden's graphing method in a linear regression model. The two methods compared can be considered identical if the slope of the regression line is equal to one and the offset of the regression line is zero (ideal case). Since in practice these values are always slightly different from ideal values, they were tested against the ideal values. If the results are consistent over the observed range, the dependence of y (method B) on x (method A) is linear:

$$y = \beta_1 . x + \beta_2 \quad (2)$$

with zero offset $\beta 2 = 0$ and the slope one $\beta 1 = 1$, ie. the line equation takes the form:

$$y = x \quad (3)$$

When evaluating the results, it was tested whether the estimates of β_1 and β_2 calculated by regression analysis were statistically significantly different (or not different) from the required values for the match of the results of both methods ($\beta_1 = 1$ and $\beta_2 = 0$). The hypothesis was tested:

H_0: $\beta 2 = 0$ and $\beta 1 = 1$, against the alternative
H_1: $\beta 2 \neq 0$ and $\beta 1 \neq 1$.

The estimated offset of the regression line β_2, calculated by the linear regression should be in the area:

$$b_2 - t_{1-\alpha/2}(n-2) \cdot \sqrt{s}_{b2} \leq \beta_2 \leq b_2 + t_{1-a/2}(n-2) \cdot \sqrt{s}_{b2} \quad (4)$$

If the calculated interval includes zero, then β_2 cannot be considered significantly different from zero.
Similarly for the slope:

$$b_1 - t_{1-\alpha/2}(n-2) \cdot \sqrt{s}_{b1} \leq \beta_1 \leq b_1 + t_{1-a/2}(n-2) \cdot \sqrt{s}_{b1} \quad (5)$$

Where: b_1 – an estimate of the slope of the regression line, b_2 – an estimate of the offset of the regression line, $t_{1-a/2}(n-2)$ – a quantile (critical value), s_{b2} – the standard deviation of the estimate of β_2, s_{b1} – the standard deviation of the estimate of β_1.

If the calculated interval also includes a unit, the β_1 region cannot be considered significantly different from one **(Meloun and Militký, 1994; Hegedűs, Čepelová, and Hegedűsová, 2015)**.

The linear regression and correlation analysis method were used to evaluate the dependence of ARA on the monitored fruit substances. The strength of the correlation relationship was evaluated by the correlation coefficient value. Correlation coefficient values of 0.7 to 0.9 were considered strong, so there is a strong interdependence between variables. Values 0.3 to 0.7 were considered for moderate and values 0.1 to 0.3 for weak correlation **(Dancey and Reidy, 2004).**

RESULTS AND DISCUSSION

The method evaluation

One of several methods of antioxidant activity determination is the DPPH method. The results of this method are often referred to as antiradical activity (ARA) since the reaction mechanism involves quenching stable DPPH radicals with the antioxidant of the substance to be tested. The first reactions of antioxidants with DPPH occur in approximately the first 30 seconds, but moderate reactions are usually complete within 2 min **(Sánchez-Moreno, Larrauri, and Saura-Calixto, 1998)**. Then the decolorization rate of the reaction mixture gradually decreases until the stabilization decrease. Ultimately, the time after which the absorbance value is read is the result of a compromise between the time required and the relative stabilization of the quenching of the free radicals DPPH and is often given for 30 min **(Gülcin, 2005)**.
The course of the quenching reaction of the DPPH colour free radicals in the reaction mixture of the extracts and the DPPH solution of several fruit species is shown in Figure 1. Some authors read the decrease in absorbance after a shorter period of antioxidant exposure to DPPH **(Vollmannová et al., 2013; Lachman et al., 2006; Jakobek et al., 2008)**.

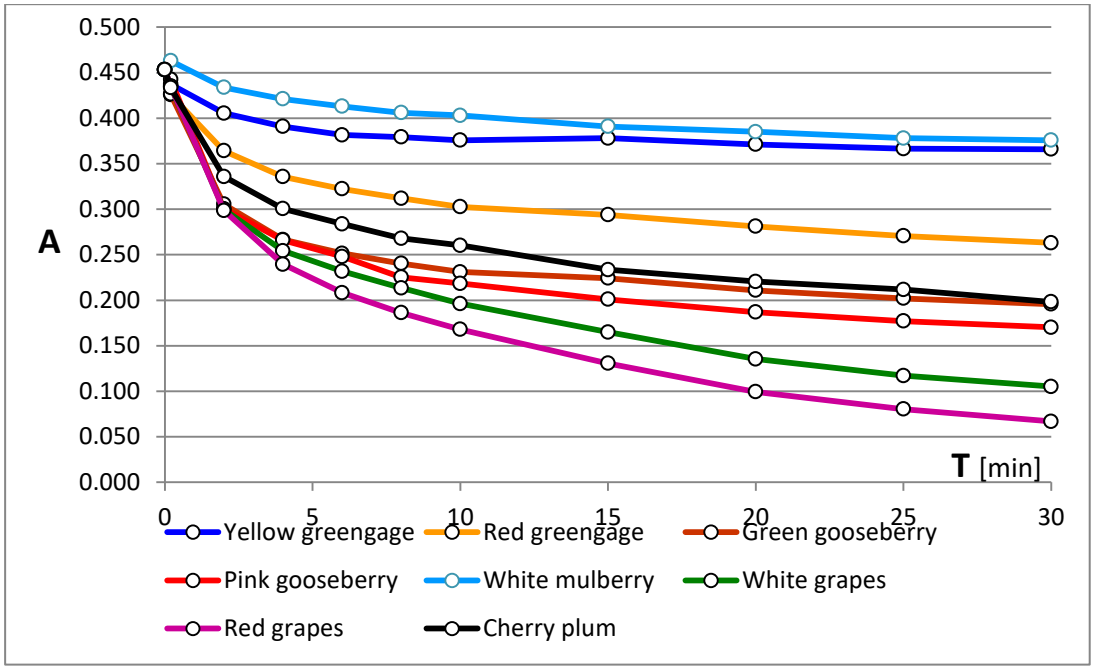

Figure 1 Examples of a decrease in the absorbance of the DPPH reaction mixture with fruit extracts.
Note: (**A** – absorbance; **T** – time).

From the decolouration of the reaction mixture according to the measurement results of Figure 1, it can be seen that in the case of several samples the colour is stabilized slowly. This may have a significant influence on the accuracy of the measurement. In the case of white and red grapes, even after 30 min the colour did not stabilize, although the changes in the decline are already apparently lower. The DPPH alcohol solution is purple and intensively absorbs output radiation at a wavelength of 517 nm (the measuring wavelength). When determining the antiradical activity of samples with a high content of anthocyanins, the question arises as to whether this fact will significantly affect the results of the determination, respectively how to adjust the method so that this problem does not occur. This is due to the intensive red to reddish-purple colour of anthocyanins, which give an intense coloration in alcohol extracts **(Apak et al., 2007)**. Several authors report the results of the assay as a measure of the reduction in DPPH staining intensity relative to a blank sample, which is a DPPH solution at the same dilution as the sample reaction mixture with DPPH **(Chang et al., 2001; Lachman et al., 2006; Vollmannová et al. 2013)**. Other authors calculate the results not relative to the blank sample, but on the initial absorbance of each DPPH reaction mixture with the sample separately **(Dawidowicz, Wianowska, and Olszowy, 2012)**, which requires more time and routine measurement, but can eliminate the problem with coloured samples. To determine the possible effect of staining the sample extract on the final result, the different fruits were analysed which gave a red-coloured alcohol extract with different shades of colour using the two methods of measurement. The results of the analyses are shown in Table 1.

After testing the hypothesis at the significance level $\alpha = 0.05$:

H_0: $\bar{x}_A = \bar{x}_B$ against the alternative

H_1: $\bar{x}_A \neq \bar{x}_B$ was found:

that after the analysis of red-coloured fruit extracts containing red-purple plant colorants (Table 1) we accept the zero hypothesis ($t_{cal.} < t_{crit.}$), thus there is no statistically significant difference between the mean ARA values obtained by two method modifications. In practice, this means that the analyses can be safely performed by correlating the results of the measured absorbance of the samples with the value of the blank, which in laboratory practice is a simpler variant. After examining the agreement of the averages, the evaluation by the Yoden graph method was also proceeded. The Yoden method statistically evaluates the equivalence of the results obtained by both adjustments over the full range of ARA values obtained. The result of the graphical analysis is shown in Figure 2.

Table 1 Antiradical activity value of samples containing red colorants measured by two method modifications.

Fruits	ARA [%/g]	
	A_{0sl}	A_0
Sour cherry 1	810	795
Sour cherry 2	750	759
Raspberry 1	547	590
Raspberry 2	440	425
Black currant	1130	1190
Red currant	544	553
Black mulberry 1	638	817
Black mulberry 2	785	817
Josta 1	646	593
Josta 2	559	677
Red greengage 1	114	76
Red greengage 2	102	88
Blueberry 1	509	465
Blueberry 2	463	423
Cherry plum 1	126	119
Cherry plum 2	116	103
Average	517	530
SD	283	309
	***t*-statistic**	
$t_{cal.}$	0.828	
$t_{crit.}$	2.131	
Degrees of freedom	27	

Note: A_0 – ARA calculated on the initial absorbance of each DPPH reaction mixture with the sample; A_{0sl} – ARA calculated on the blank absorbance value; ***SD*** – standard deviation; *t*-statistic: t_{cal} – calculated value; $t_{crit.}$ – critical value.

The graphical presentation of the results of the analyses with both modifications of the method shows that the results obtained by both modifications of the method give a regression line that is almost identical to the ideal course of the regression line. Thus, the graphic assessment does not point to the possibility of a proportional error.

The demonstration of the existence of a proportional error was based on estimates of the parameters $\beta 1$ and $\beta 2$. Linear regression dependence parameter estimates are:
Estimation of the slope of the regression line, b_1 is 1.015,
Regression line offset estimate, b_2 is -8.166,
Coefficient of determination, R^2 is 0.962,
The number of parallel analyses, n_A, and n_B, was 44.

By solving relations (4) and (5) we found out when the estimate of the guideline b_1 can be considered equal to 1 and the estimate of the displacement b_2 equal to 0. Since in practice we always work with a certain variance of the tested parameters, in our case the confident areas are important, in which the values of β_1 and β_2 lie with the chosen significance level $\alpha = 0.05$. The limits of the regression line parameters β_1 and β_2 are:
Lower limitation of the regression line, $\beta_{1\min.}$ is 0.950,
Upper limitation of the slope of the regression line, $\beta_{1\max.}$ is 1.079,
Lower limitation of the regression line offset, $\beta_{2\min.}$ is -34.813,
Upper r limitation of the regression line offset, $\beta_{2\max.}$ is 18.481.

The results obtained show that the calculated interval for the offset β_2 also includes zero, so the segment b_2 cannot be considered significantly different from zero. Similarly, the calculated interval for β_1 includes one, so the estimate of slope b_1 cannot be considered significantly different from one. For our case, this means that there is no statistically significant difference between the compared method modifications under the conditions described and the concentration range of the assay. In practice, this means that coloured fruit extracts do not affect the result of the analysis simply because of their colour. This can be explained by the fact that the anthocyanin colours that are reddish carriers are also important antioxidants. They can quench free DPPH radicals and thus stain themselves.

Effect of fruit components on ARA

It was assumed that the value of antiradical activity will be influenced by several fruit substances. Therefore, it was considered a complicated mechanism when monitoring. Since various phenolic compounds as well as ascorbic acid are present in fruits, the ultimate effect was likely the result of their interactions.

Effect of ascorbic acid on ARA

Ascorbic acid is present in various fruit species and its content is at different concentrations. It is capable of quenching free DPPH radicals. It is sometimes used as an equivalent to express the antiradical activity of a sample **(Du Toit, Volsteedt and Apostolides, 2001)**. The influence of ascorbic acid on the determined ARA value was investigated on the example of analysed fruits. The results of the analyses are shown in Figure 3 as the dependence of ARA on the determined amount of ascorbic acid in all samples analysed, ie. samples containing and without red plant colorants.

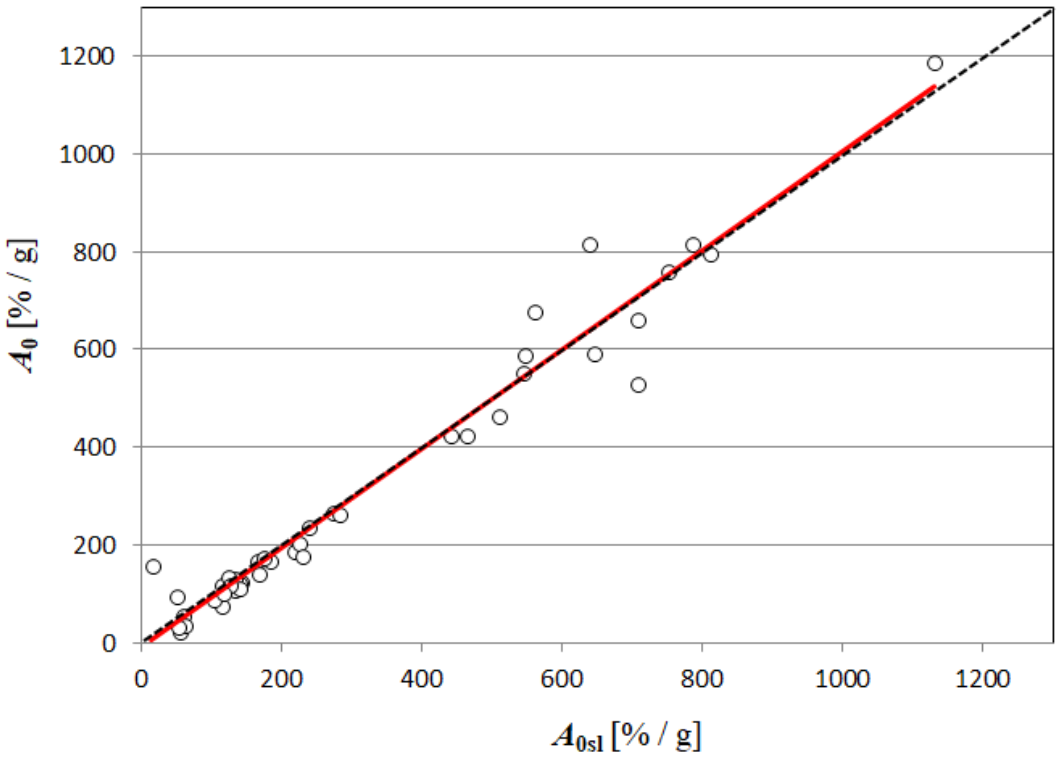

Figure 2 Graphical evaluation of the determined ARA values.
Noter. ARA values calculated on the blank absorbance value (A_{0s1}) and the compared ARA values calculated on the initial absorbance of each DPPH reaction mixture with the sample (A_0). ------- ideal regression line with zero offset and unit slope; ——— regression line calculated from the determined ARA values.

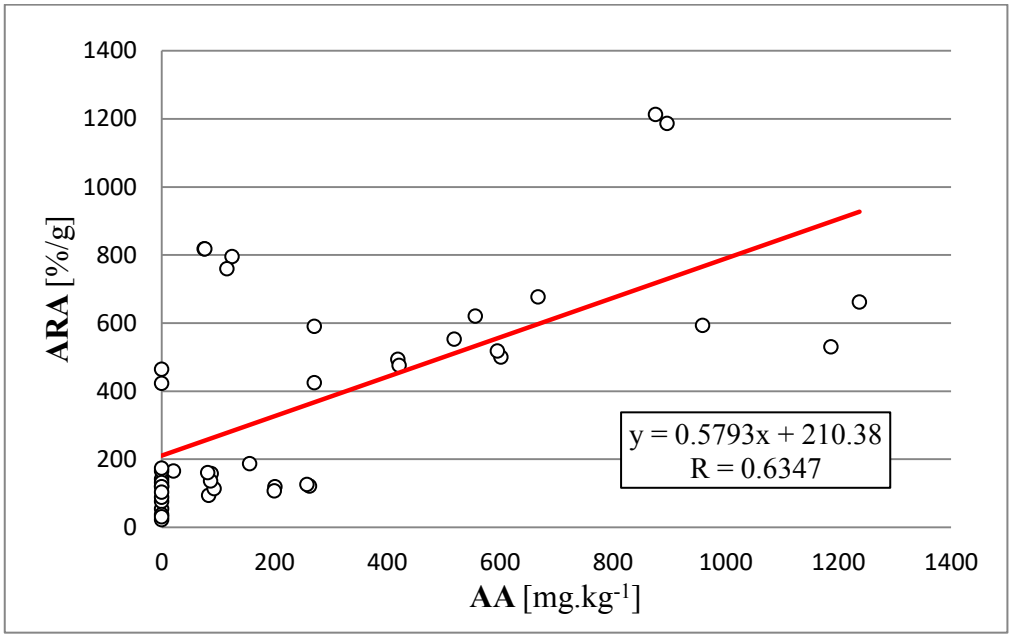

Figure 3 Dependence of the determined antiradical activity (ARA) value on the L-ascorbic acid (AA) content for all samples analysed.

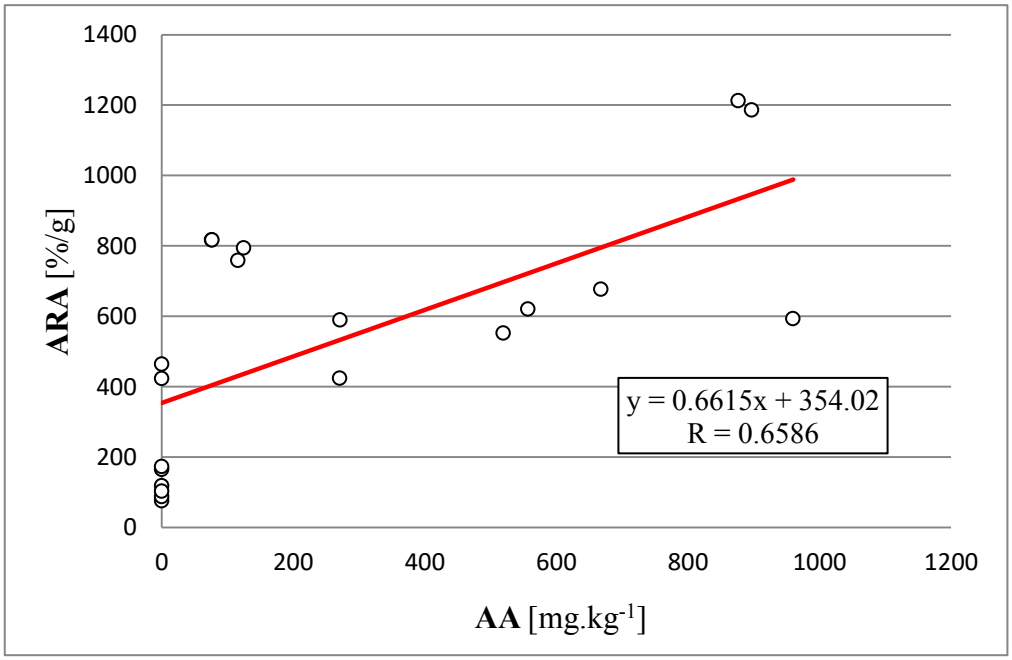

Figure 4 Dependence of the determined antiradical activity (ARA) value on L-ascorbic acid (AA) content for samples containing red plant colorants.

The correlation dependence between monitored values was searched from the analysed data. It was assumed that the higher the content of ascorbic acid, the more it would show its antioxidant properties, thus the higher the ARA value. Data analysis for all analysed samples shows that the calculated correlation coefficient is 0.6347, indicating a slight correlation between the ascorbic acid content and the corresponding ARA value.

Based on the fact that in addition to ascorbic acid, many other substances, among them red anthocyanin colorants affect the ARA value, the analysed samples were divided into two groups for further investigation of this dependence. In one group, samples with a red-coloured extract from the presence of anthocyanin colorants were included. In the second group were sampled with extract without red plant colorants. Figure 4 shows the dependence of the value determined by ARA on the content of L-ascorbic acid (AA) for red-coloured sample extracts.

The analysis data shows that the calculated correlation coefficient is 0.6586. As in the case of all samples, it shows a slight correlation between the ascorbic acid content and the corresponding ARA value.

The correlation dependence of ARA on the content of ascorbic acid for samples with extract without red colorants is shown in Figure 5.

Analysis of the data for samples with no colorant extract shows that the calculated correlation coefficient is 0.9038. This indicates a strong correlation between the ascorbic acid content and the corresponding ARA value. Based on the correlation dependencies observed, ascorbic acid is the predominant antioxidant component for samples containing no anthocyanin colorants. When investigating the antiradical activity of small kinds of fruits **Jakobek and Seruga (2012)** also found the significant antiradical potential of ascorbic acid, which was weaker than the potential of some polyphenols. **Hegedűs et al. (2010)** also found a correlation between antiradical activity and ascorbic acid in apricot, but the correlation relationship was weaker than for polyphenols.

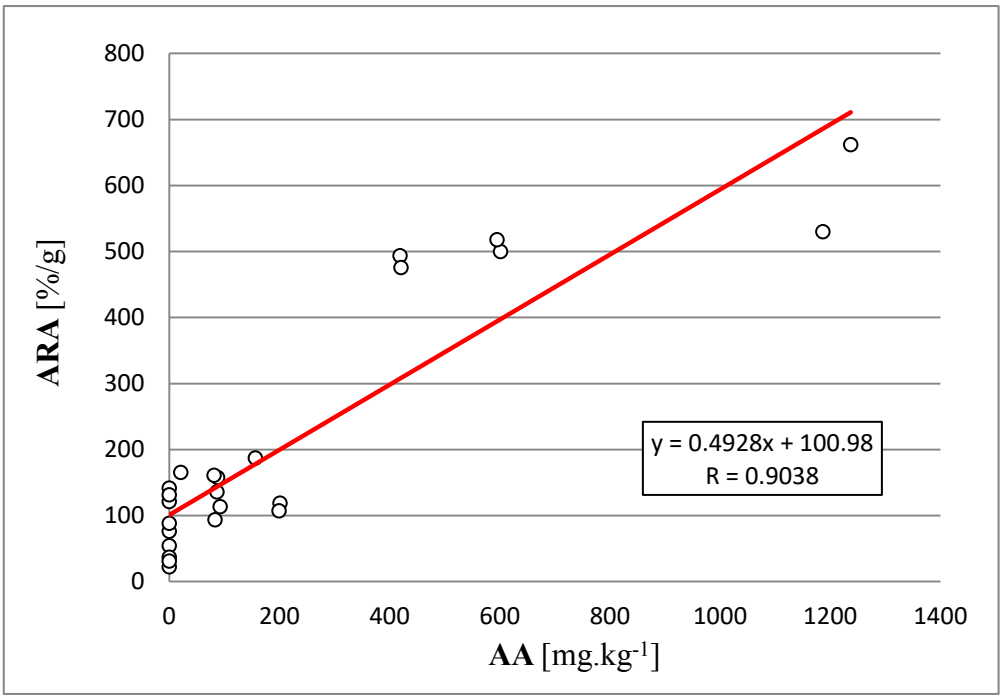

Figure 5 Dependence of the determined antiradical activity (ARA) value on the L-ascorbic acid (AA) content for extracts without red plant colorants.

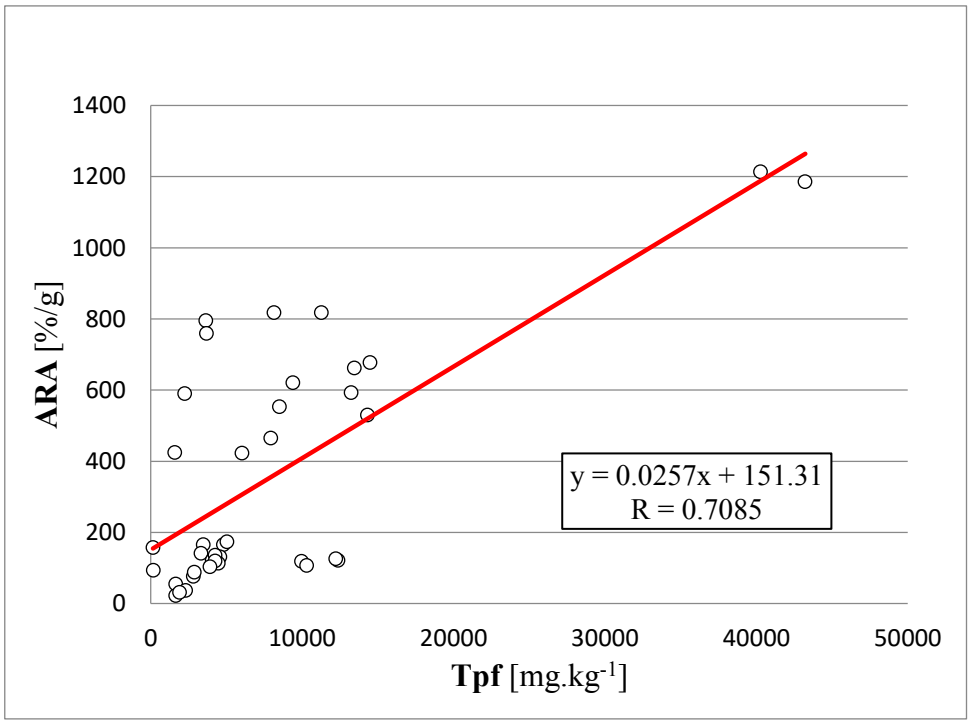

Figure 6 Dependence of the determined antiradical activity (ARA) on the total phenol content (Tph) for all samples analysed.

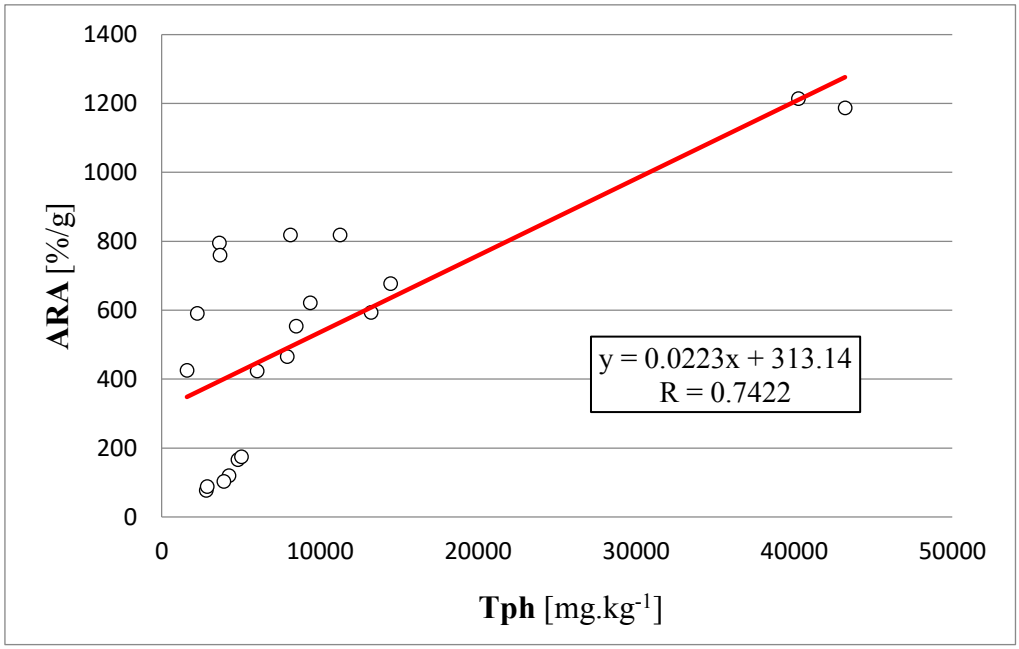

Figure 7 Dependence of determined antiradical activity (ARA) value on the total phenol content (Tph) for samples stained red.

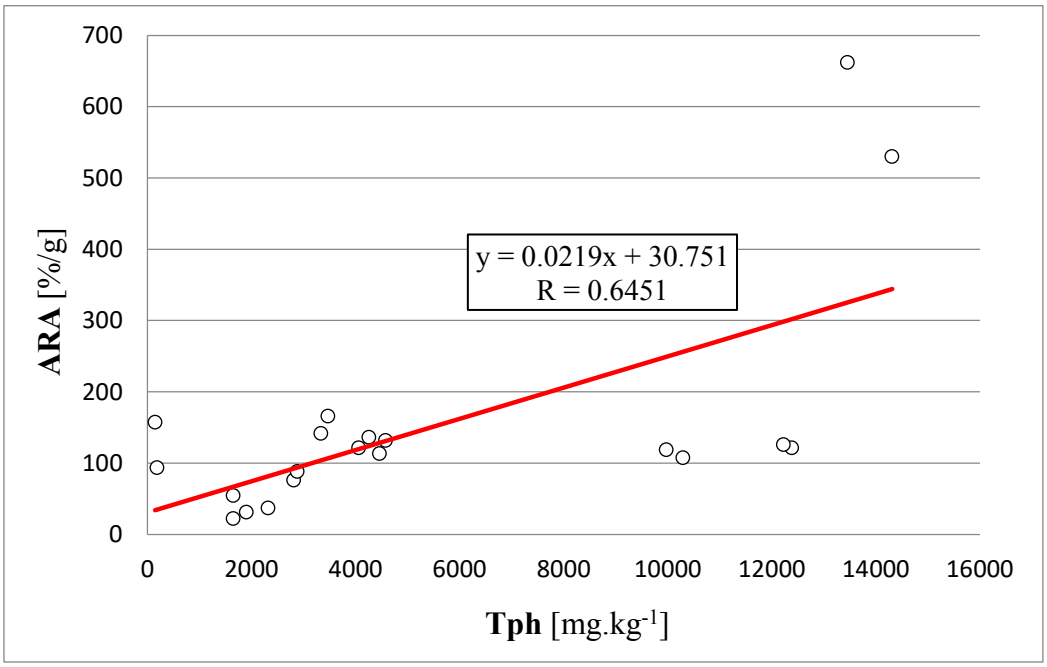

Figure 8 Dependence of determined antiradical activity (ARA) value on the total phenol content (Tph) for samples without the presence of red colorants.

Effect of total phenols on ARA

Another group of substances with antioxidant properties, we have studied due to their proven effect of ARA are phenolic compounds. These compounds are present in fruits as a large group of flavonoids, including a large number of flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. They are characterized by strong antioxidant effects, similarly, like ascorbic acid, they quench free DPPH radicals. The effect of total phenols on ARA based on our analysis is shown in Figure 6.

Based on the correlation analysis, the results show a strong correlation between the polyphenol content and the corresponding ARA value (correlation coefficient value is 0.7085). Given the wide variety of phenolic compounds, it is understandable that this results in a milder correlation dependency than that of ascorbic acid.

To obtain more detailed information on the effect of phenolic compounds that carry red colouring of the fruit (anthocyanin colorant group), samples containing these compounds were removed from the study and subjected to further analysis. The results are shown in Figure 7.

Based on the regression analysis of the results shown in Figure 7, it is evident that in the case of red-coloured fruits, the strength of the dependence of the ARA on the total phenol content does not differ significantly from that observed for all samples analysed together.

It was expected a significant difference in dependence by comparing the group of red-coloured fruits to the group of red colorants free fruits. Dependence results for fruit species without red plant colorants are shown in Figure 8.

Comparing the results of the correlation analysis for fruit species with and without red colorants, it is clear that the correlation coefficient value for red fruits is higher than for the group of fruits without red colorants (0.7422 vs. 0.6451). The anthocyanin colorants present have a greater influence on the ARA value and indicate a strong correlation dependence.

Similar results were obtained by **Jakobek and Seruga (2012)** when monitoring the effect of total phenols on ARA in small kinds of fruits. They found a strong correlation between the total phenol content and ARA, while the greatest effect on ARA values was found for anthocyanins.

A significant effect of phenols on the antioxidant activity is also described by **Gramza-Michałowska and Człapka-Matyasik (2011)**. They observed the highest levels of antioxidant activity in the aronia, which contained the most polyphenols and anthocyanins when considering the different fruit species. A high correlation between polyphenols and ARA has also been shown by other authors **(Jakobek et al., 2007; Hegedűs et al., 2010; Rinaldi et al., 2013)**.

CONCLUSION

Statistical evaluation of the results of the analyses by Youden's graph method revealed that the analysed extracts of red fruit species, although absorbing the light used at 517 nm as a DPPH free radical solution, did not affect the final ARA value determined. This means that coloured fruit extracts do not affect the result of the analysis by this method due to their colour. Investigation of the effect of the evaluated fruit compounds revealed a strong correlation between the content of ascorbic acid and ARA in samples containing no red plant colorants. For samples containing red colorants, the correlation was slight. The opposite trend was found in the case of fruit species with the presence of red colorants. It was proven a strong dependence of ARA on the total phenol content.

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METHODS FOR DETERMINING THE BOTANICAL ORIGIN OF HONEY

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ABSTRACT

The demand for monofloral, original, and special (functional) kinds of honey, or those with geographical indication, is forecast. At the same time, there is a need to improve the methods for determining the botanical and geographical origin of honey. The purpose of the research was to select and apply a variety of techniques for identifying the botanical origin of honey for its correspondence to acacia species. Samples of honey from the Kyiv, Odesa, and Dnipro regions extracted in the spring and summer period were used in the research. Organoleptic, physicochemical, NMR spectrometry, and advanced melissopalynology methods were applied. The tests were carried out at the laboratories of the Department of Certification and Standardization of Agricultural Products, NULES, Ukraine; the Ukrainian Laboratory of Quality and Safety of Agricultural Products; and the Bruker BioSpin GmbH company (Germany). According to the research results, the requirements for acacia honey were met by the organoleptic method for samples B1 and B2; by the physicochemical method for A0 and A2; by NMR spectroscopy for not a single sample, all being assessed as polyfloral; and by pollen analysis for B1 and B2. The conducted studies confirm the need for a comprehensive approach to the identification of the botanical origin of honey for its conformity to acacia species. There is a need to review the physicochemical indicators for the compliance of honey with the acacia species obtained in Ukraine. After all, even the modern NMR spectrometry technique indicated that the specially fabricated sample that did not contain acacia pollen grains was acacia honey. Identification of the botanical origin of monofloral honey, in particular acacia, should be carried out in the following sequence: pollen analysis (by dominant pollen grains), safety (presence of antibiotics, pesticides), physicochemical parameters according to international requirements, organoleptic parameters.

Keywords: acacia honey; evaluation; method; NMR spectrometry

INTRODUCTION

According to **Global Industry Analysts Inc. USA (2016)**, the global honey market is projected to reach 2.4 million tonnes by 2022. **Carreck (2018)** believes that much of this growth is due to the demand for monofloral and special (functional) kinds of honey, or those that have a specific geographical region of origin. That is the relevance of our research, along with the improvement of the methods for determining the botanical and geographical origin of honey, methods of analysis and discrimination of individual varieties of product, its safety, and quality.

At the moment, the current regulatory and technical documentation in different countries of the world governing the safety and quality of honey is not harmonized. There are differences between European legislation and Codex Alimentarius standards. Also, different countries maintain up-to-date quality criteria that do not comply with the Codex or EU directives on honey. The necessity to establish national requirements for each country (harmonized with international ones) is mainly due to the lack of provisions regarding the physicochemical characteristics of monofloral honey and the declaration of its geographical origin **(Thrasyvoulou et al., 2018)**.

There are currently two documents in Ukraine governing the safety and quality of honey, **DSTU 4497:2005 (2007)** and **Nakaz MinAPK No. 330 (2019)**. Therefore, the search for methods that allow the main botanical varieties and geographical species of Ukrainian honey to be studied is a relevant area for analysis and subsequent research.

It has been established **(Ulloa et al., 2013; Lenhardt et al., 2015; Machado De-Melo et al., 2018; Maione, Barbosa and Barbosa, 2019)** that the organoleptic and physicochemical characteristics of honey vary depending on botanical and geographical origin, as well as climatic conditions, processing and storage. And other bee products as well **(Ivanišová et al., 2015)**.

It is relevant to use honey as a biomarker to collect environmental information, identify sources of environmental contamination and assess soil, water and air pollution **(Machado De-Melo et al., 2018)**. One of the first and most common methods of determining the botanical origin of honey is melissopalynology, that is, a microscopic analysis of the pollen contained in honey shows the nectar content of certain plants.

Maione, Barbosa and Barbosa (2019) consider that it is still used as one of the most accurate methods for qualitative or quantitative analysis of the content of pollen grains in honey. Along with this, there are several modern techniques. The botanical origin of honey is identified by PARAFAC (parallel factor) analysis of excitation emission matrix fluorescence spectra **(Lenhardt et al., 2015)**. Emissions of phenolic compounds and Maillard reaction products reveal the greatest difference among honey varieties of different botanical origin. Falsified honey samples with 100% sensitivity to variety compliance can be detected using the partial least squares discriminant analysis (PLSDA) classification model based on PARAFAC models. Among the honey samples tested, PLSDA identifies linden with a difference of 0.5%, acacia with 10%, and sunflower and polyfloral meadow honey with differences of about 20% compared to the results of pollen analysis. The disadvantage of the method is the large discrepancy between the results obtained even within the same honey variety, which indicates the need for its improvement.

Soares et al. (2015) proposed the use of DNA-based methods for the identification of botanical honey species. For this, five DNA extraction methods were used (NucleoSpin Plant Kit (methods A and B) and DNeasy Plant Mini Kit, as well as internal CTAB and Wizard-based methods). The results demonstrated that the Wizard method had the best performance in terms of DNA quality. The disadvantages of DNA-based methods are their cost and unsuitability to industrial conditions of use.

Today, the use of analytical methods is becoming widespread. Mainly, this concerns nuclear magnetic resonance (NMR) spectroscopy for the authentication of honey (botanical and geographical origin).

Siddiqui et al. (2017) consider NMR spectroscopy to be sufficiently powerful and accurate, and therefore suitable for creating prints for honey of different origin for their further comparison. In Romania, studies on stable isotopes, selected as representative discrimination parameters of different botanical or geographical origin of honey, have been conducted using isotope ratio mass spectrometry (IRMS) and site-specific natural isotope fractionation measured by nuclear magnetic resonance (SNIF-NMR) methods. The authors **(Dinca et al., 2015)** confirmed the high efficiency of the method but emphasized the need to create an informative base for prints of honey of different botanical origin.

Gok et al. (2015) established the predictive ability of Fourier transform infrared (FTIR) spectroscopy and chemometrics to determine the botanical composition of honey. **Cozzolino, Corbella and Smyth (2011)** proposed spectroscopic methods in the infrared (IR) wavelength range of the electromagnetic spectrum to evaluate and control the composition of honey. The use of IR spectroscopic technologies has its place in the determination of the botanical origin of honey by comparing the spectra of the mid-infrared (MIR) spectrum.

Etzold and Lichtenberg-Kraag (2008) used the most common types of honey for calibration, and classification models were obtained by calibration of principal components analysis (PCA) and properly graded FTIR concerning the physicochemical and sensory properties of the selected samples.

To improve honey discrimination, **Corvucci et al. (2015)** proposed the use of FT-micromanaging spectrography and multivariate analysis. At the same time, successful results using the PCA model have also been achieved by other authors. This method is currently being refined and enhanced.

Modern sensory methods include the use of an electronic nose (e-nose) in multivariate analysis and selection of sensors to detect the botanical origin and determine honey quality. **Huang et al. (2015)** first implemented three sensor selection algorithms, namely, uninformation variable elimination (UVE), successive projections algorithm (SPA) and competitive adaptive reweighted sampling (CARS), which were applied to analyse the e-nose fingerprints of honey. Three different sampling modes were tested for the classification of monofloral honey: static headspace sampling (SHS), solid-phase microextraction (SPME) and inside needle dynamic extraction (INDEX). The last two showed better ability to remove volatile components. In subsequent experiments, preference was given to the SPME sampling mode, which proved to be more accurate.

Ampuero, Bogdanov and Bosset (2004) found a positive correlation between e-nose and pollen analysis of honey. Discriminant function analysis (DFA) was also conducted using an e-nose based on a mass spectrometer when the DFA diagram indicated a significant separation of honey odours from other odour sources **(Hong et al., 2011)**. Thus, the e-nose method can be used to identify honey but needs further refinement.

Ulloa et al. (2013) investigated a method for determining the botanical origin of honey using sensory synthesis of impedance electronic language (e-language) and optical spectroscopy (UV-Vis-NIR), namely, PCA and cluster analysis (CA).

In 2016, the mathematical method for identifying honey was first described. **Gan et al. (2016)** considered the use of PLSDA, a support vector model (SVM), and an interval partial least squares model (iPLS). The results showed that the spectra and sensors classified the botanical origin of honey quickly and accurately, and the overall accuracy for the calibration and forecasting set was 100% for e-nose and electronic tongue (ET) analysis using the SVM model and near-infrared (NIR) and MIR analysis using the iPLS model. At the same time, overall accuracy for calibration and forecasting sets was above 96% in PLSDA NIR, MIR and ET models. The results showed that ET is more suitable for detecting botanical falsification of honey. However, there is a need to create calibration and forecasting sets for each sample of honey produced in the world. This makes it impossible to use this method in practice.

Son et al. (2019) investigated whether the zymography of nectar chitinases is a potential marker for determining or validating the botanical origin of honey. However,

zymography is the first examination of the activity of nectar enzyme in honey. This method is also being refined.

Chekryga, Niczievskaya and Borodaj (2019) have proposed a method for determining the botanical origin of honey, which is to use a natural drop of a honey sample without pre-treatment. According to the authors, the advantage of the proposed method over others is that when using it there is no deformation of pollen grains, which are in a natural state, including their spatial location. However, the proposed method casts doubt on the quantitative evaluation of pollen grains and the reliability of the analysis results due to the uneven arrangement of grains of different weights in the thickness of honey.

Sensory data obtained from ET and e-nose histograms of honey colour show a high discriminatory ability to determine the origin of honey. Therefore, many authors **(Machado De-Melo et al., 2018; Pascual-Maté et al., 2018)** believe that PCA, discriminant analysis (DA) and CA are the best methods for performing the experimental and predictive method for determining the origin of honey. PCA and DA continue to be favoured due to their ease of application and interpretation, while machine learning algorithms are more complex for modelling and the use of classifiers. Nevertheless, the use of both machine learning algorithms and PCA-DA models have achieved excellent results for discrimination of the origin of honey. Finally, a common trend is the use of hybrid methods that combine multivariate analysis of data and methods **(Peng Kek et al., 2017; Machado De-Melo et al., 2018; Pascual-Maté et al., 2018)**.

Ballabio et al. (2018) performed a comparative evaluation of methods for determining the botanical origin of honey. Thus, IR, NIR, Raman spectroscopy, PTR-ToF-MS and e-nose methods were applied to samples of common botanical varieties of honey. The best results were obtained with the synthesis of the NIR method and Raman spectroscopy, as well as PTR-ToF-MS. The accuracy of the final model was 99% on the test specimens and 100% on the calibration.

In Ukraine, due to a lack of the necessary equipment, only various pollen analysis techniques are used to identify the origin of honey. Therefore, the purpose of our work was to select and apply a variety of techniques to identify the botanical origin of honey for its correspondence to acacia variety.

Scientific hypothesis

According to many scientists, modern methods of researching honey can replace the classical methods of determining its botanical origin, such as pollen analysis and organoleptic evaluation. In studies, we expect to refute the view that NMR spectroscopy allows determination of the falsification of acacia honey without the use of melissopalynological research, provided that the geographical origin of the honey is not known.

As a result, one of the samples was falsified in a sophisticated manner and NMR spectroscopy did not reveal this. Also, two specimens were identified as acacia monofloral honey by pollen and organoleptic analyses. Instead, NMR spectroscopy showed that one of these samples did not meet the requirements for acacia honey by physicochemical parameters.

MATERIAL AND METHODOLOGY

Biological material

The research used honey samples obtained from the Kyiv, Odesa and Dnipro regions obtained in the spring and summer that could be realized on the market as acacia. Honey from bees was collected in March – May 2018. The samples were stored in glass containers at +15 to +20 °C away from sunlight until the start of the research in August 2018.

Honey samples

Sample A0 was obtained from nectar mixed with sugar syrup by bees (stored in the fall), which they processed into honey, put in cells and sealed; the sample was obtained from bees in the form of honey in March 2018 (Kyiv region). A1 – honey centrifuged from bee honeycombs in May 2018 (Kyiv region); A2 – in June 2018 (Kyiv region); B1 – in May 2018 (Odesa region); B2 – May 2018 (Dnipro region). Acacia bloom in Ukraine is due in May.

Organoleptic analysis

The analysis was conducted at the laboratories of the Department of Certification and Standardization of Agricultural Products, National University of Life and Environmental Sciences (NULES) of Ukraine, with the use of the methodology and requirements specified according to **DSTU 4497:2005 (2007)**.

Physicochemical analysis

The analysis was conducted at the Ukrainian Laboratory of Quality and Safety of Agricultural Products.

Chemicals

All chemicals were of analytical grade and were purchased from LLC "NVP"ALFARUS" (UA).

Mass fraction of water

The mass fraction of water was determined on an LR-01 laboratory refractometer (Maselli Misure s.p.a., Italy) using a standardized technique according to **DSTU 4497:2005 (2007)**.

Hydroxymethylfurfural, diastasis and proline

Hydroxymethylfurfural, diastasis and proline were investigated with a KFC-3 photocalorimeter (Russia) using standardized methods according to **DSTU 4497:2005 (2007)**. All the techniques have been pre-elaborated and described in detail for acacia honey of different geographical origin **(Adamchuk, Suchenko and Akulonok, 2019)**.

NMR spectrometry

The analysis was carried out at the laboratories of Bruker BioSpin GmbH **(Bruker, 2020)** (Germany) using Avance Neo and Benchtop NMR Fourier 80 devices (Germany) and a technique which allows the acquisition of a fingerprint to confirm the authenticity of the product or reveal adulteration by addition of sweetener **(Schievano et al., 2020)**. All NMR samples were prepared by dissolving ~240 mg honey in phosphate buffer solution (KH_2PO_4 in

D_2O), adjusting the honey (mg)/buffer (mL) ratio to exactly 240 mg.mL^{-1}. The pD was carefully adjusted from 133 to 4.40.

Botanical origin

Botanical origin was determined according to the adapted harmonized methods of melissopalynology **(Von Der Ohe et al., 2004)** using a Sigeta Biogenic Led Trino Infinity microscope (China) with 400× and 2000× magnification based in the laboratories of the Department of Certification and Standardization of Agricultural Products, NULES of Ukraine with the use of **DSTU 4497:2005 (2007)**. Identification of plants was carried out according to the methodology and experience of the team of the international network AgroBio*Net* **(Brindza et al., 2018)**.

Statistical analysis

Basic statistical analysis was carried out using SAS programming packages (SAS System V9.2). Correlation coefficients were calculated by CORR analysis **(SAS, 2009)**.

RESULTS AND DISCUSSION

In organoleptic research on honey for compliance with acacia variety requirements, evaluations were made by colour, taste, aroma, consistency, crystallization and the presence of signs of fermentation and mechanical impurities. The results of the organoleptic research are shown in Table 1.

According to the results of the organoleptic evaluation, only samples B1 and B2 met the requirements for acacia honey. Sample A0 had the lowest compliance (14%).

Among the physicochemical indicators were those that indicate the naturalness and enzymatic activity of honey – diastase number, and proline and hydroxymethylfurfural content. Also, they have values that are different from other honey varieties. The results are shown in Table 2.

According to the conducted studies, in terms of the mass fraction of water, hydroxymethylfurfural and diastasis number, all tested honey samples met the requirements. The highest proline content was found in the falsified sample A0 – 483.7 ±0.36 mg.kg^{-1}, which is 76% and 54% higher than the other samples from Kyiv region, which did not correlate with acacia varieties by organoleptic indicators; and 71% higher than the honey samples that corresponded to acacia species in organoleptic indicators (B1 and B2).

In general, according to the physicochemical parameters investigated, samples A0 and A2 corresponded to the requirements of **DSTU 4497:2005 (2007)** for acacia honey. At the same time, according to the requirements of another current regulatory document, which is used today for the circulation of the product in the country and its export, all the research samples met the established criteria.

Thus, according to the results of honey studies, which were carried out by standardized methods, the data obtained differed by organoleptic and physicochemical parameters in accordance with the acacia variety. By the latter, two samples, one of which was a pre-prepared falsification (A0), fully met the requirements for acacia honey compliance. This speaks about the need to review complex methods of evaluating honey to identify it as an acacia variety.

Table 1 Organoleptic honey research.

No.	Indicator		Honey sample				
	Name	Characteristic	A0	A1	A2	B1	B2
1.	Color	Colorless, light yellow, transparent	–	–	–	+	+
2.	Crystallization	Absent	–	+	+	+	+
3.	Signs of fermentation	Prohibited	–	–	–	+	+
4.	Taste	Sweet, delicate, without any foreign flavors	–	–	–	+	+
5.	Aroma	Very weak, no odours	–	–	–	+	+
6.	Consistency (liquid)	A small amount of honey is left on the spatula, which quickly drains into small drops	–	+	–	+	+
7.	Mechanical impurities	Prohibited	+	+	+	+	+
Compliance with acacia honey variety, %			14	43	29	100	100

Note: (+) – meets the requirements for acacia honey; (–) – does not meet the requirements for acacia honey.

Table 2 Physicochemical honey research (n = 2).

Sample	Mass fraction of water, no more than, %		HMF, no more than, mg.kg^{-1}		Diastasis, no less than, Goethe units		Proline, no less than, mg.kg^{-1}	Compliance with the DSTU requirements for acacia honey, %
Standard^{D}	**18.5^{HG}**	**21^{FG}**	**10**	**25**	**15**	**5**	**200**	
Standard^{N}	**20**		**40**		**3**		**100**	
A0	16.6		7.9 ±0.19		20.3 ±0.11		483.7 ±0.36	100
A1	15.0		7.7 ±0.00		19.1 ±0.16		118.2 ±0.73	75
A2	16.2		7.4 ±0.19		31.0 ±0.05		220.5 ±0.36	100
B1	16.6		2.2 ±0.10		10.0 ±0.05		141.2 ±0.73	75
B2	16.8		1.6 ±0.10		9.6 ±0.10		139.2 ±0.10	75

Note: D –standard defined in **DSTU 4497:2005**; N –standard defined in **Nakaz MinAPK No. 330** dated 19.06.2019; HG – highest grade; FG – first grade; HMF – hydroxymethylfurfural.

Table 3 NMR spectroscopy of sugars in honey, g.100g^{-1}.

No.	Indicator	A0	A1	A2	B1	B2
1.	Glucose + fructose	74.1	74.4	73.8	75.6	78.2
2.	Fructose/glucose	1.34	1.54	1.38	1.68	1.30
3.	Fructose	42.4	45.1	42.7	47.4	44.2
4.	Glucose	31.7	29.3	31.1	28.2	33.9
5.	Saccharose	0.7	2.5	1.1	3.5	0.6
6.	Turanose	1.7	2.7	2.4	2.9	1.9
7.	Maltose	2.4	2.7	2.6	3.3	2.2
8.	Raffinose	0.2	0.3	0.2	0.2	0.1

Table 4 Honey-Profiling^{TM} profile for sugars according to NMR spectroscopy.

Indicator	A0	A1	A2	B1	B2
Glucose + fructose	62.8 86.2	62.8 86.2	62.8 86.2	62.8 86.2	62.8 86.2
Fructose/glucose	0.88 1.58	0.88 1.58	0.88 1.58	0.88 1.58	0.88 1.58
Fructose	32.9 45.4	32.9 45.4	32.9 45.4	32.9 45.4	32.9 45.4
Glucose	25.6 43.9	25.6 43.9	25.6 43.9	25.6 43.9	25.6 43.9
Saccharose	<0.5 2.9	<0.5 2.9	<0.5 2.9	<0.5 2.9	<0.5 2.9
Turanose	0.4 3.0	0.4 3.0	0.4 3.0	0.4 3.0	0.4 3.0
Maltose	<0.5 3.6	<0.5 3.6	<0.5 3.6	<0.5 3.6	<0.5 3.6
Melezitose	<1.0 1.1	<1.0 1.1	<1.0 1.1	<1.0 1.1	<1.0 1.1
Maltotriose	<1.0 g.100g^{-1} in reference dataset	<1.0 g.100g^{-1} in reference dataset	<1.0 g.100g^{-1} in reference dataset	<1.0 g.100g^{-1} in reference dataset	<1.0 g.100g^{-1} in reference dataset
Gentiobiose	<0.3 0.4	<0.3 0.4	<0.3 0.4	<0.3 0.4	<0.3 0.4
Raffinose	<0.1 0.4	<0.1 0.4	<0.1 0.4	<0.1 0.4	<0.1 0.4
Mannose	<0.05 0.23	<0.05 0.23	<0.05 0.23	<0.05 0.23	<0.05 0.23

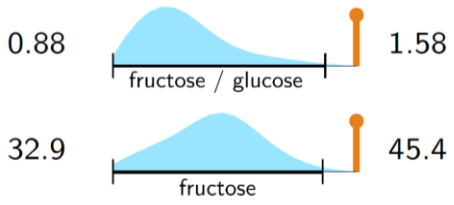

Figure 1 Discrepancy between the honey profile of sample B1 and the reference NMR database.

The next step was the evaluation of honey samples using a modern NMR spectroscopy technique that is widely used in internationally accredited laboratories. Its core consists of the complex analysis of physicochemical parameters, on the basis of which the profile of the honey is programmatically constructed and its botanical origin determined. NMR spectroscopy of honey made it possible to analyse 12 indicators for sugar, including the ratio of fructose to glucose (Table 3).

According to our data, the ratio of fructose to glucose (FG) ranged from 1.30 to 1.68. According to studies of the acacia honey profile by other scientists **(Marghitas et al., 2010)**, it is known that the FG index ranges from 1.43 to 1.57 (the content of acacia pollen grains in honey was in the range from 20.0% to 30.2%). According to other data **(Oddo et al., 2004)**, the highest average FG value for acacia honey from Europe is 1.61 (715 samples tested). At the same time, honey from Ukraine is accepted for export as acacia if FG is more than 1.5.

According to **Marghitas et al. (2010)** and **Schievano et al. (2020)**, sucrose and maltose content may also indicate botanical origin. Thus, for acacia honey, **Marghitas et al. (2010)** found that the sucrose content in acacia varieties ranged from 0.59 to 2.50 g.100g^{-1}, and the maltose content from 2.31 to 3.07 g.100g^{-1}. In addition, **Nakaz MinAPK No. 330** allows a sucrose content of not more than 10 g.100g^{-1}, and maltose is not specified. Thus, for sucrose, the honey we studied met the requirements for acacia variety compliance. The presence of sugars other than those listed in Table 3 was investigated, but their quantitative values were beyond the limit of determination. Among these were melezitose (limit of determination – 1 g.100g^{-1}), maltotriose (1 g.100g^{-1}), gentiobiose (0.3 g.100g^{-1}) and mannose (0.05 g.100g^{-1}).

The Honey-Profiling^{TM} profile was built from the set of indicators using software for NMR spectroscopy (Table 4).

Honey-Profiling^{TM} indicates a deviation from the norm for certain indicators. The profile of honey sample B1 contains red marks, which indicates the need for a more detailed analysis of individual indicators for this honey sample or re-analysis with NMR spectrometry (Figure 1).

If a negative result is obtained again, such samples are considered falsified. **Schievano et al. (2020)** recommend further CSSF-TOCSY experiments for refining the analysis to detect the sugar profile of honey and its falsification with sugar. According to their latest results, the level of fructose in the honey of European origin ranges from 36.7 to 49.4 g.100g^{-1}, which coincides with our results. NMR spectroscopy allows analysis of the acid composition of honey (Table 5).

Table 5 Acid NMR spectroscopy, mg.kg^{-1}.

		Basic criteria				
No.	**Organic acids:**	**A0**	**A1**	**A2**	**B1**	**B2**
1.	Citric acid	79	71	61	72	58
	Amino acids:	**A0**	**A1**	**A2**	**B1**	**B2**
2.	Alanine	11	7	8	<LOQ	8
3.	Proline	560	318	694	248	307
4.	Valine	11			<LOQ	
	Additional parameters of fermentation, processing and origin					
5.	Acetic acid	14	18	15	11	<LOQ
6.	Ethanol	21	6		<LOQ	
7.	Lactic acid	41	13	55	<LOQ	11
8.	Formic acid	25	27	50	10	21
9.	Pyruvic acid	15	<LOQ	21		<LOQ
10.	Succinic acid	17	7	21	5	7

Note: <LOQ – below the limit of quantification.

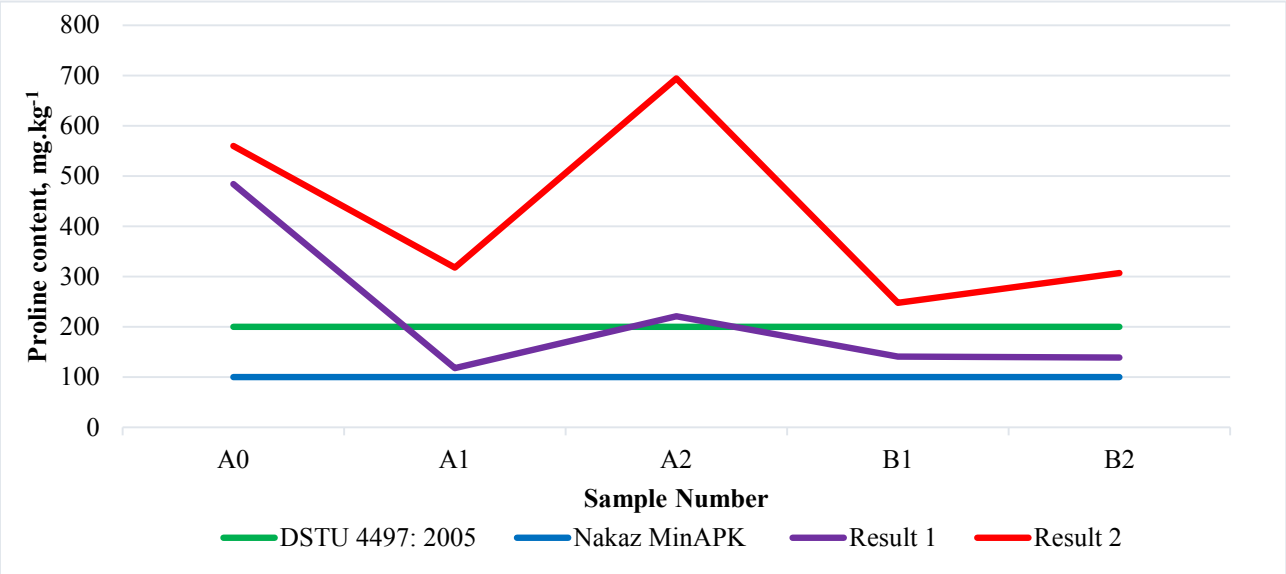

Figure 2 Proline in the investigated honey samples, mg.kg^{-1}: result 1 – by the method standardized according to **DSTU 4497:2005** "Natural honey. Specifications"; result 2 – by NMR spectrometry.

Among organic acids, citric acid was found in the range from 58 to 79 mg.kg^{-1}; malic (limit of detection – 100 mg.kg^{-1}) and quinic acids (300 mg.kg^{-1}) were beyond the limit of quantification qualification. High proline content was characteristic of all samples tested. The highest was found in sample A2 – 694 mg.kg^{-1}. In general, the results from proline studies differed from those obtained using standardized techniques. Some of the amino acids were beyond quantification, namely aspartic acid (detection limit – 150 mg.kg^{-1}), glutamine (200 mg.kg^{-1}), leucine (40 mg.kg^{-1}), tyrosine (50 mg.kg^{-1}) and phenylalanine (100 mg.kg^{-1}).

A comparison of the proline indicator results obtained according to the requirements of **DSTU 4497:2005 (2007)** and the criteria for the honey composition according to **Nakaz MinAPK No. 330 (2019)** is shown in Figure 2.

In addition, special substance markers are used to identify honey from individual geographical regions. For example, for manuka honey (New Zealand), it is methylglyoxal. The markers in the test specimens were beyond quantification, namely 3-phenyl lactic acid (quantification limit – 300 mg.kg^{-1}), dihydroxyacetone (20 mg.kg^{-1}), kynuric acid (60 mg.kg^{-1}), methylglyoxal (30 mg.kg^{-1}) and shikimic acid (80 mg.kg^{-1}).

Thus, NMR spectrometry makes it impossible to determine honey as regional (geographical definition of honey from Ukraine).

Also, additional indicators were used to control the parameters of fermentation, processing and origin (Table 5). Among these, acetic acid was detected in four test specimens, ranging from 11 to 14 mg.kg^{-1}; ethanol in two – from 6 to 21 mg.kg^{-1}; lactic acid in four – from 11 to 55 mg.kg^{-1}; formic acid in all samples in the range from 10 to 50 mg.kg^{-1}; pyruvic acid in two – from 15 to 20 mg.kg^{-1}; and succinic acid in all samples – from 5 to 21 mg.kg^{-1}. Other substances were beyond detection, namely 2,3-butanediol (detection limit – 20 mg.kg^{-1}), 5-hydroxymethylfurfural (5 mg.kg^{-1}), acetoin (20 mg.kg^{-1}) and fumaric acid (5 mg.kg^{-1}). All of them give an opportunity to evaluate the safety and quality of honey in terms of physicochemical composition, but do not give an understanding of its botanical origin.

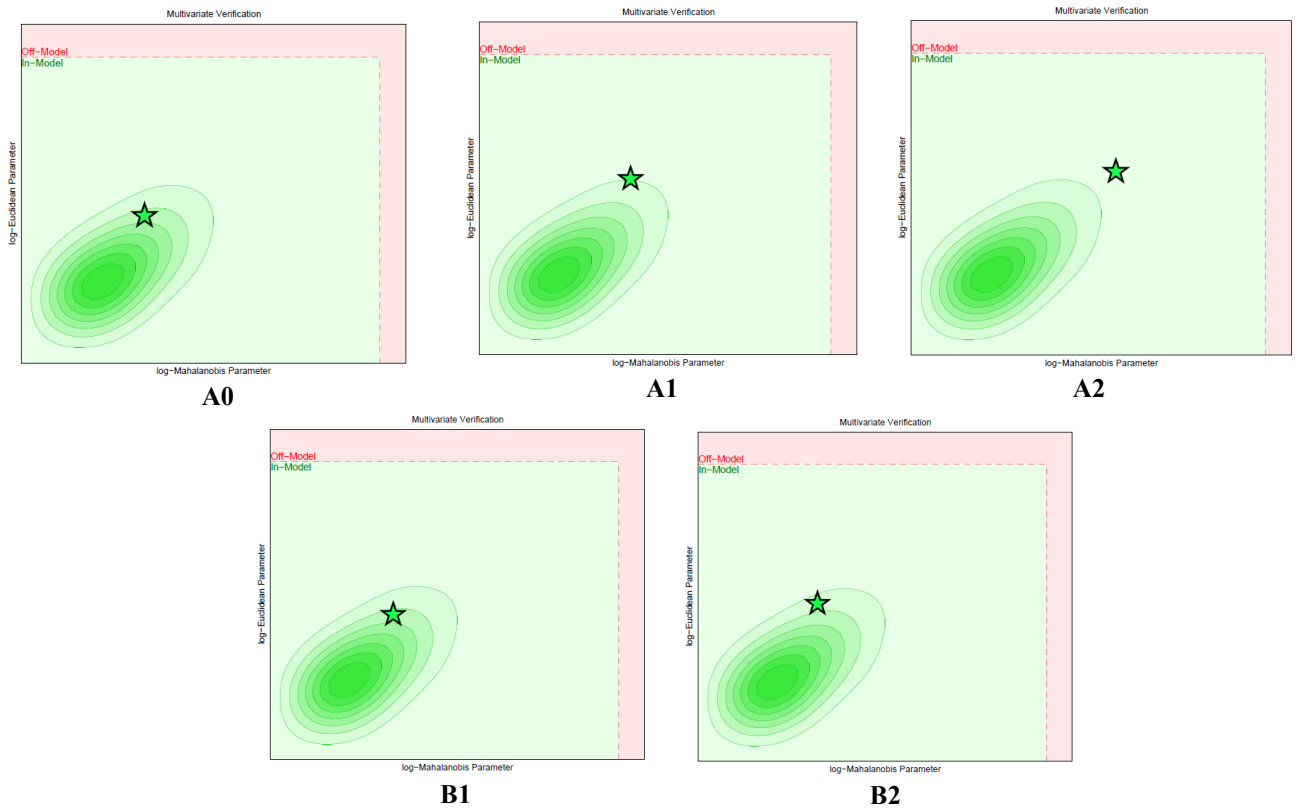

Figure 3 Multivariate verification models of investigated honey.

Upon completion of the test, software verification analysis was applied and graphical models of the studied honey samples were automatically constructed (Figure 3).

Displaying the results in the form of multivariate verification models gives us a visual understanding of how much a honey sample corresponds to the declared botanical origin by the complex of physicochemical parameters studied. Thus, samples A0 and B1 are closest to the centres of correspondence (intense green colour). For this, A0 is a falsification prepared in advance.

Schievano et al. (2020) claimed that no significant differences were found in the sugar profiles in a group of European (i.e. Italian and Eastern European) acacia honeys. This allowed them to assume the minor influence of different geographical origin on the sugar profile. However, in our opinion, the sugar profile should not change significantly if the honey is natural and not falsified with sugar.

At the same time, **Schievano et al. (2020)** found that the sugar profile of Chinese honey purchased on the Italian market did not match. They commented on the significant decrease in endogenous sugar, which they believe is the result of feeding bees with sugar syrups during the honey harvest period. This also confirms our previous conclusions regarding the confirmation of falsification.

However, in our sample A0, which was falsified using the method of feeding bees with sugar before winter, NMR spectrometry did not detect falsification.

Along with this, there are known methods **(del Campo et al., 2016; Spiteri et al., 2016)**, which allow the identification in honey of some species of plants, such as eucalyptus, heather, lavender, orange, thyme and rosemary. For this purpose, the authors used analysis of carboxylic acids (acetic, formic, lactic, malic and succinic acids), amino acids (alanine, phenylalanine, proline and tyrosine), carbohydrates (α- and β-glucose and fructose), ethanol and hydroxymethylfurfural.

Gerhardt et al. (2018) presented results where they were able to distinguish three varieties of honey, namely canola, acacia and honeydew honeys, with a predictive accuracy of 98.6% using additional HS-GC-IMS profiling.

Schievano et al. (2020) asserted that melissopalynological analysis and SCIRA also do not reveal any particular anomalies in Chinese acacia honey samples. This is probably due to new methods of falsification. According to our previous research **(Adamchuk, Suchenko and Akulonok, 2019)**, pollen analysis of honey using harmonized methods **(Von Der Ohe et al., 2004)**, which we adapted to the laboratory conditions of Ukraine, is reliable in the botanical identification of honey. Thus, in the 30 acacia honey samples tested, we found 20% to 30% *Robinia pseudoacacia* pollen grains. This indicates compliance with the current standards for acacia monofloral honey in Ukraine **(Nakaz MinAPK No. 330, 2019)**. In this case, the tested honey samples also met the requirements for diastasis and hydroxymethylfurfural.

For evaluation of the botanical origin of honey, the NMR spectrometry method proved to be ineffective for our samples. All honey was programmatically identified as polyfloral. Therefore, we applied the harmonized method of melissopalynology **(Von Der Ohe et al., 2004)**, which we adapted to the conditions of the laboratory at the Department of Certification and Standardization of Agricultural Products, NULES of Ukraine; and improved the way of counting and identifying pollen grains of different types of plants widespread in Ukraine.

The results of pollen analysis are shown in Table 6.

Table 6 Botanical origin of honey.

Sample	Pollen grains, %			
	Predominant ≥20%	**Secondary 10 – 20%**	**Minor ≤10%**	**Including ≤1%**
A0	-	Asteraceae *Barbarea vulgaris*	*Draba nemorosa* *Lamium album* *Trifolium repens* *Bistorta officinalis*	*Convolvulus arvensis* *Agrimonia eupatoria* Anemophilic species of plants
A1	-	*Robinia pseudoacacia* (17%) *Acer negundo* *Aesculus hippocastanum* *Ajuga reptans*	*Acer platanoides* *Acer tataricum* *Lamium album* *Salix* spp. *Tilia* spp. *Clinopodium vulgare* *Lycopus* spp.	Asteraceae Anemophilic species of plants
A2	-	*Robinia pseudoacacia* (16%) *Brassica napus* *Centaurea* spp.	*Ficaria verna* *Swida alba* *Ballota nigra* *Melilotus officinalis* *Tilia* spp. *Rosa canina* *Salix alba* *Catalpa bignonioides*	*Fragaria vesca* Fruit tree *Juglans* spp. Anemophilic species of plants
B1	*Robinia pseudoacacia* **(33%)**	Fabaceae *Acer* spp.	*Caragana arborescens* *Bunias orientalis* *Barbarea vulgaris* *Nasturtium officinale* *Brassica napus* *Salix* spp. *Lamium purpureum* *Primula* spp.	*Anemone* spp. Anemophilic species of plants
B2	*Robinia pseudoacacia* **(39%)**	*Gleditsia triacanthos* *Elaeagnus argentea*	*Salix* spp. *Vicia cracca* *Melilotus officinalis* *Quercus* spp. *Rhus hirta* *Lotus corniculatus* *Veratrum lobelianum* *Clinopodium vulgare* *Gagea* spp. *Allium ursinum* *Taraxacum officinale* *Cornus mas* *Geum rivale* *Ajuga reptans* *Scrophularia vernalis* *Tussilago farfara* *Amorpha fruticosa*	*Lamium purpureum* *Lamium maculatum* *Lamium galeobdolon* *Ribes* spp. Anemophilic species of plants

According to the results of the research, samples B1 and B2 correspond to the acacia variety by botanical origin. They contain 33% and 39% acacia (*Robinia pseudoacacia*) pollen grains, respectively. According to **Nakaz MinAPK No. 330 (2019)**, the only normative document regulating the criteria for monofloral honey varieties, for acacia honey, the *Robinia pseudoacacia* pollen grain content should be at least 20%.

Samples A1 and A2 also contained acacia grains, 17% and 16%, respectively. Along with this, sample A1 also contained a large amount of pollen from maple (*A. negundo*, *A. platanoides*, *A. tataricum*), horse-chestnut (*A. hippocastanum*) and bugleweed (*A. reptans*). Sample A2 contained a significant amount of pollen from rapeseed (*B. napus*) and several species of *Centaurea* spp., indicating the activation of flight activity of bees on field honey plants after the end of acacia flowering. The presence of willow (*Salix* spp.) pollen grains indicates a failure of adherence to acacia honey production technology, which is based on the use of additional cases of the 145-frame hive. Pollen from willow, which is an early pollen source, can only be present in the hive body.

The conducted studies confirm the need for a comprehensive approach to the identification of the

botanical origin of honey for its conformity to the acacia variety. There is a need to revise the physicochemical indicators regarding the conformity of honey obtained in Ukraine to the acacia variety. After all, even the modern NMR spectrometry technique indicated that sample A0, which did not contain acacia pollen grains and was specially fabricated, was acacia honey.

CONCLUSION

Identification of the botanical origin of monofloral kinds of honey, in particular acacia, should be carried out in the following sequence: pollen analysis (by dominant pollen grains), safety (presence of antibiotics, pesticides), physicochemical parameters according to international requirements, organoleptic parameters. The methods of determination and requirements for physicochemical indicators of honey require revision, improvement and harmonization.

In the future, the safety of acacia honey in terms of the content of contaminants that may be caused by anthropogenic environmental load requires analysis and research. Also, there is a necessity to find markers that will indicate the geographical origin of Ukrainian kinds of honey and thus protect them from falsification from other countries.

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ANTIMICROBIAL POTENTIAL OF DIFFERENT MEDICINAL PLANTS AGAINST FOOD INDUSTRY PATHOGENS

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ABSTRACT

Work aimed to investigate the antimicrobial activity of medicinal plants against selected species of food industry pathogens *in vitro* conditions. The detection of antibacterial properties was examined by the disc diffusion method and the method of the minimum inhibitory concentrations (MIC). The cultivation of microorganisms after the 24 h was performed by disc diffusion method. Petri dishes have grown at 37 °C in which the Mueller - Hinton agar and application it to the sterile paper disc impregnated with the extract. The thickness of the resulting inhibition zone was measured with a ruler after completion of the culture. After the preparation of bacteria and extracts of certain concentrations of a subsequently added to wells microplates we use the method of the minimum inhibitory concentration (MIC) which was conducted out as the second measurement, and we took the readings absorbance spectrophotometer at 570 nm using the Glomax plate spectrophotometer. We found out, that *Equisetum arvense* demonstrated the largest zones of inhibition to the tested Gram-positive and Gram-negative bacteria. The greatest antimicrobial activity achieved *Equisetum arvense*, *Urtica dioica,* and *Taraxacum officinale* against *Salmonella enterica* subsp. *enterica* CCM 3807 and *Yersinia enterocolitica* CCM 5671. *Equisetum arvense* and *Taraxacum officinale* was the most effective against *Escherichia coli* CCM 2024 and the least effective were *Tussilago farfara* and *Mentha piperita w*ith using the method of minimum inhibitory concentrations.

Keywords: medicinal plants; antibacterial effect; Gram-positive and Gram-negative bacteria; disc diffusion method; MIC

INTRODUCTION

Salvia officinalis belongs to the largest genus of the family Lamiaceae containing approximately 900 different species worldwide. Some of them can be used as an ingredient in the kitchen, others have been used in the cosmetics industry, and their essential oils are also used as ingredients in various fragrances. Analysis of essential oils of different sage species has shown the most important components, which are made up of substances such as eucalyptol, borneol, thujone, or camphor. Eucalyptol or 1,8-cineol is a natural organic compound. These are cyclic ether and monoterpenoid. Positive properties of killing to leukemia cells have been found *in vitro* **(Baser et al., 1993; Baser et al., 1997; Ahmadi and Mirza, 1999)**.

The essential oil of sage extract as reported **Hammer et al. (1999) and Marino et al. (2001)** has inhibitory properties against the following bacteria: *Bacillus cereus, Bacillus megatherium, Bacillus subtilis, Aeromonas hydrophila, Aeromonas sobria, Klebsiella oxytoca, Candida albicans, Shigella, Salmonella,* and *Cryptococcus*. Small inhibition zones against *Escherichia coli* and *Staphylococcus aureus* have also been observed. The antimicrobial activity of sage *in vitro* was determined using a minimum inhibitory concentration (MIC) and achieved a higher activity against Gram-positive bacteria. The most sensitive of these were *S. epidermidis* (MIC = 12.5 $\mu g.mL^{-1}$), *B. cereus* and *B. subtilis* (MIC = 25 $\mu g.mL^{-1}$). In the case of Gram-negative bacteria, only significant activity was demonstrated against *E. coli, S. typhi* and *K. pneumoniae*, which showed the lowest MIC values (50 $\mu g.mL^{-1}$). It was moderately active against *P. vulgaris* and *S. enteritidis* (MIC = 100 $\mu g.mL^{-1}$) **(Tenore et al., 2011)**.

Melissa officinalis belongs to the family Lamiaceae. It is characterized by having 2 to 8 cm soft, slightly hairy leaves, with older, larger leaves having a heart shape **(Bown, 2001)**. Lemon balm as a medicinal plant has proven antimicrobial and antiviral effects. In addition to these effects, anti-fungal **(Darouche et al., 2006)**, immunomodulatory **(Fang et al., 2005)**, and antioxidant effects comparable to tocopherol have also been shown. In addition to soothing effects, lemon balm extract has anti-bloating effects, convulsions, antibacterial and antiviral effects, as well as anti-inflammatory, antioxidant and neuroprotective effects **(Dastmalchi et al., 2008; Pereira et al., 2009)**.

Mentha x piperita (*Lamiaceae*) is generally used for various therapeutic purposes. It comes from Europe and

the Middle East, is widespread in Brazilian culture, and can grow in all areas of the country thanks to its modesty. Essential oils show a strong bactericidal effect, in particular against *Escherichia coli*. When inhaled oil was used concomitantly with multiple drugs in patients with lung tuberculosis, the number of bacilli was demonstrably reduced **(Dejani et al., 2014)**. Mint contains a large amount of fragrant (0.1 – 1%) and essential oils. These are mainly monoterpenes, the main components of which are menthol (29 – 48%), menthone (20 – 31%), menthofuran (6.8%), and their derivatives (isomenthone, neomenthol, menthyl acetate, and pulegone). In menthol and peppermint oil, fungicidal and antiviral activity against *Candida albicans, Aspergillus albus*, dermatophytic microscopic fungi, and Herpes simplex virus has been demonstrated **(Spirling and Daniels, 2001)**.

Equisetum arvense (Equisetaceae) finds its use in internal use for flushing in bacterial and inflammatory diseases of the urinary tract, kidney sand, gout, and rheumatic diseases; as concomitant treatment in chronic lung diseases, osteoporosis, varicose veins, and immunosuppression. The gilt tea is used to relieve gastric mucosal irritations and heartburn. As the age in the human body increases, the silicon content decreases, so the use of this plant is particularly meaningful in geriatrics **(Bühringová, 2010)**. In determining the antimicrobial activity by the disk diffusion method, the essential oil from the gilt showed strong activity against all strains tested. With the highest inhibition zone, values were found against Gram-negative *S. enteritidis* (35 mm) and *K. pneumoniae* (37 mm). The antimicrobial activity of the essential oil of horsetail oil can be attributed to the presence of various substances, in particular phenol, monoterpenes, and thymol. Also, the combination of thymol and 1,8-cineol may result in an important synergistic fungicidal effect **(Vaťľák et al., 2014)**.

Taraxacum officinale belongs to the family Asteraceae. It has a beneficial effect on human health due to its antioxidant and antiallergic properties. The root extract can be used as a probiotic *in vitro* **(Yan et al., 2011)**. A very important part of the plant is the group of sesquiterpene lactones (have anti-inflammatory and anti-cancer effects), phenylpropanoids, polysaccharides and triterpenoids of saponins. The major components of sesquiterpene lactones can often be found in the form of glycosides. These compounds include taraxacine, taraxacerin, dihydrolactucin, ixerin **(Schütz et al., 2006a, b)**. Leaves and dandelion root have beneficial effects in digestion and are considered bitter digestive stimulants. Diuretic effects have also been shown. They probably share a high content of daisies. The root is significant due to mucoprotective, prebiotic, hypoglycemic, and immunostimulatory effects. The leaves in turn have diuretic and anti-inflammatory effects **(Trojanová et al., 2004)**.

Tussilago farfara has found its application in the treatment of cough, bronchitis, and asthmatic diseases. Phytochemical studies have shown that the flower contains several types of metabolites, including essential oils, sesquiterpenes, triterpenes, flavonoids, and phenylpropanoids **(Li et al., 2013)**. Studies have shown that coltsfoot has marked pharmacological and antagonistic effects and also has antioxidant and antimicrobial activity **(Gao et al., 2008)**. The antimicrobial activity of the coltsfoot *in vitro* was determined using the disk diffusion method. The highest antibacterial activity of *Tussilago farfara* was found on Gram-positive bacteria *Lactobacillus rhamnosus* (6.67 ±1.53 mm) and lower on yeast *Saccharomyces cerevisiae* (1.67 ±0.58 mm) **(Kačániová et al., 2013)**.

Urtica dioica (Urticaceae) has been known in Europe and has been used as a medicinal plant for over 2000 years. The leaf and the root are used for these purposes. Leaf extract has been found to be of use in the symptomatic treatment of arthritis, arthrosis, and rheumatic problems, as well as a diuretic for inflammatory diseases of the urinary tract and bladder and overall detoxification of the body. The whole part of the plant can be used for various purposes such as food, feed, medicines, cosmetics, biodynamic agriculture, and textile production **(Bodros and Baley, 2008)**. Clinical studies have shown that the juice obtained from this plant has a diuretic effect in patients suffering from congestive heart failure. It helps in digestion and promotes milk production of nursing mothers. It provides short-term pain relief and is therefore also used to treat rheumatism. It has also been used in the treatment of arthritis, urinary tract diseases, respiratory diseases, body cleansing, etc. It contains silicic acid, which has diuretic effects, chlorophyll with anti-inflammatory and disinfectant properties, anti-bleeding tannins, and glucokinins, which lower blood sugar **(Bisht et al., 2012)**.

Scientific hypothesis

The different antimicrobial effect exists among analysed medicinal plants for individual food industries pathogens. Antimicrobial activity to pathogenic bacterial strains of food industries is variable for individual medicinal plant species.

MATERIAL AND METHODOLOGY

Plant material

Extracts from 7 different medical plants, obtained from Nitra region, were used to demonstrate antimicrobial activity against selected pathogens. The plant parts such as stems, leaves, and flowers were left to dry in a well-aired dark place at room temperature. Ten grams of dried plants were crushed and extracted with 100 mL of ethanol. Extracts from the plants were leached for two weeks at room temperature and subsequently, ethanol was evaporated by vacuum evaporator (Stuart RE300DB, UK). The samples were stored in the freezer at -20 °C.

Tested strains of microorganisms

For determination of antimicrobial activity 5 bacterial species were purchased from Czech Collection of Microorganisms (CCM, Brno). Three of selected strains were Gram-negative (*Salmonella enterica* subsp. *enterica* CCM 3807, *Yersinia enterocolitica* CCM 5671, *Escherichia coli* CCM 2024) and two strains were Gram-positive (*Staphylococcus aureus* subsp. *aureus* CCM 2461, *Listeria monocytogenes* CCM 4699).

Table 1 Antimicrobial activity with disk diffusion method in mm (mean ±*SD*).

Medicinal plants	*Escherichia coli* CCM 2024	*Salmonella enterica* subsp. *enterica* CCM 3807	*Yersinia enterocolitica* CCM 5671	*Staphylococcus aureus* subsp. *aureus* CCM 2461	*Listeria monocytogenes* CCM 4699
Tussilago farfara	11.33 ±0.58	13.33 ±0.58	12.37 ±0.58	5.33 ±0.58	4.67 ±1.15
Equisetum arvense	14.67 ±0.58	14.33 ±0.58	15.33 ±0.58	6.33 ±0.58	6.67 ±0.58
Melissa officinalis	12.33 ±1.53	10.67 ±0.58	10.67 ±1.53	4.33 ±0.58	5.33 ±0.58
Urtica dioica	8.67 ±1.15	10.33 ±1.15	11.33 ±0.58	4.67 ±0.58	4.67 ±0.58
Taraxacum officinale	8.33 ±0.58	8.33 ±1.53	10.67 ±0.58	3.67 ±0.58	4.00 ±1.00
Salvia officinalis	7.33 ±0.58	9.33 ±1.53	9.33 ±0.58	3.33 ±0.58	4.67 ±0.58
Mentha piperita	7.67 ±0.58	9.33 ±0.58	8.67 ±0.58	4.33 ±0.58	3.33 ±0.58

Disk diffusion method

Five bacterial cultures grown overnight at 37 °C Muller Hinton broth (MBH) were diluted with distilled water to the turbidity 0.5 according to McFarland standard (measured on densilameter). Petri dishes with Mueller-Hinton agar were covered by 100 μL of cell culture with use of L-shaped cell spreader. Petri dishes were dried in a thermostat.

For each bacteria strain were prepared 9 discs with 6 mm diameter (7 discs with medicinal plants, 1 positive control and 1 negative control). Discs were soaked in plant extracts with use of sterile tweezers and afterwards were placed on Petri dishes with cell cultures. Disc with distilled water was used as negative control and disc with antibiotic (gentamycin for G^+ bacteria and tobramycine for G^- bacteria) was used as positive control.

Prepared samples were incubated for 24 hours at 37 °C. After incubation period, diameter of each inhibition zone was measured in 3 directions, and average size of inhibition zone was calculated.

Minimum inhibitory concentration

The microdilution method is carried out in 0.5 mL microtiter plates. There was A 96 well microtiter plate used in the assay. A stock solution of various concentrations of the medicinal plant is prepared for each well of the plate. There are 12 wells in row A – H into which we pipet 0.5 mL of Mueller – Hinton broth culture medium. The plant extract was pipetted in the next step from row A – H of the first column. Plant extracts were prepared according to the desired concentration and diluted with dimethylsulfoxide. The extracts were pipetted with the given microorganisms with a concentration of 0.5 McFarland. Using an automatic eight-channel pipette with a preset value of 100 μL, the suspension from the wells of the first column was transferred to the second column. We repeated the procedure until the concentration was reduced in this way. The whole plate concentration was measured with an absorbance spectrophotometer at 570 nm using the Glomax plate spectrophotometer. The cultivation proceeded from 16 to 18 hours. After its completion, we again measured concentration throughout the plate, and from the pre-and post-cultivation readings, we calculated the differences in absorbance. It can be stated that the minimum inhibitory concentration and its value had the lowest extract concentration at which inhibitory bacterial growth was detectable.

Statistical analysis

Mean and standard deviation with Excel were used for the disk diffusion method. Using obtained absorbance before and after the analysis, we were able to express the differences in absorbance between the measurements as a set of binary values. These values were assigned to exact concentrations. The following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05. For this statistical evaluation probit analysis in Statgraphics software was used for minimal inhibitory concentration.

RESULTS AND DISCUSSION

Several studies have described different biological effects of *Equisetum arvense* L., *Tussilago farfara, Melissa officinalis, Urtica dioica, Salvia officinalis* and *Mentha piperita* extract or tea with natural extract, such as antioxidant, anti-inflammatory, antibacterial, antifungal, vasorelaxant, neuro and cardio protectors **(Dos Santos et al., 2005; Sandhu et al., 2010; Salehzadeh et al., 2014; Salih, 2014; Rabbani et al., 2015; Lee et al., 2019)**, and antiproliferative properties **(Yamamoto, Inoue and Hamako, 2004; Četojevic-Simin et al., 2010; Stanojevic et al., 2010)**. Disk diffusion method (Table 1) indicated that *E. coli, S. enterica,* and *Y. enterocolitica* were more sensitive to extracts from selected medicinal plants than *L. monocytogenes* and *S. aureus.*

The most significant antimicrobial effect was observed at *Equisetum arvense* that exhibited the largest inhibition zones against all bacteria. Considerable results were observed at *Y. enterocolitica, E. coli,* and *S. enterica* with sizes of inhibitory zones 15.33 ±0.58 mm, 14.67 ±0.58 mm and 14.33 ±0.58 mm respectively. Also, *L. monocytogenes* and *S. aureus* showed the highest sensitivity against *Equisetum arvense*.

Tussilago farfara and *Melissa officinalis* also showed significantly larger inhibition zones, while *Salvia officinalis* and *Mentha piperita* seemed to be the least effective. Overall this test indicated that Gram-negative bacteria are more susceptible to the antimicrobial activity of medicinal plant extracts.

According to the minimum inhibitory concentration test (Table 2), slight variations in antimicrobial sensitivity were observed against the disk diffusion method.

Table 2 Minimum inhibitory concentration of medicinal plants in μg.mL^{-1}.

Medicinal plants		*Escherichia coli* CCM 2024	*Salmonella enterica* subsp. *enterica* CCM 3807	*Yersinia enterocolitica* CCM 5671	*Staphylococcus aureus* subsp. *aureus* CCM 2461	*Listeria monocytogenes* CCM 4699
Tussilago farfara	**MIC50**	> 25.6	19.15	19.15	> 25.6	25.60
	MIC90	> 25.6	20.36	20.36	> 25.6	28.57
Equisetum arvense	**MIC50**	**9.59**	**9.59**	**9.59**	12.80	12.80
	MIC90	**10.20**	**10.20**	**10.20**	14.29	14.29
Melissa officinalis	**MIC50**	12.80	12.80	19.15	25.60	12.80
	MIC90	14.29	14.29	20.36	28.57	14.29
Urtica dioica	**MIC50**	12.80	**9.59**	**9.59**	12.80	12.80
	MIC90	14.29	**10.20**	**10.20**	14.29	14.29
Taraxacum officinale	**MIC50**	**9.59**	**9.59**	**9.59**	19.15	19.15
	MIC90	**10.20**	**10.20**	**10.20**	20.36	20.36
Salvia officinalis	**MIC50**	19.15	**9.59**	10.85	> 25.6	19.15
	MIC90	20.36	**10.20**	16.67	> 25.6	20.36
Mentha piperita	**MIC50**	18.03	**9.59**	25.60	25.60	> 25.6
	MIC90	29.07	**10.20**	28.57	28.57	> 25.6

However, the same trend with higher sensitivity against plant extract was observed in a group of Gram-negative bacteria *E. coli, S. enterica,* and *Y. enterocolitica*. Also, the most conspicuous growth inhibition was caused by *Equisetum arvense* extract with the lowest detected inhibitory concentration at MIC 50: 9.59 μg.mL^{-1} and MIC 90: 10.20 μg.mL^{-1} for Gram-negative bacteria and MIC50: 12.8 μg.mL^{-1} and MIC90: 14.29 μg.mL^{-1} for Gram-positive bacteria (*L. monocytogenes* and *S. aureus*). This observation corresponded with the previous test.

In addition, according to MIC test, also *Taraxacum officinale* and *Urtica dioica* extracts achieved comparable values of cell growth inhibition as *Equisetum arvense.* On the other side, *Mentha piperita* and *Tussilago farfara* seemed to be the least effective.

From the perspective of bacteria, the most sensitive to the antimicrobial activity of all tested medicinal plant extracts was *S. enterica* followed by another Gram-positive bacteria *E. coli* and *Y. enterocolitica* that were slightly more resistant especially to plant extracts with inferior antimicrobial effectivity.

A great interest in biologically active substances of plant origin increased in recent years. Many of these substances demonstrated an antimicrobial effect **(Essawi and Srour, 2000)**.

Kačániová et al. (2013) tested the antimicrobial activity of coltsfoot (*Tussilago farfara*) against selected species of microorganisms: *Escherichia coli* CCM 3988, *Enterococcus raffinosus* CCM 4216, *Staphylococcus epidermis* CCM 4418, *Lactobacillus rhamnosus* CCM 1828, *Pseudomonas aeruginosa* CCM 1960, *Serratia rubidaea* CCM 4684, and *Saccharomyces cerevisiae* CCM 8191. Ethanol extract of coltsfoot with two methods, disk diffusion method, and minimal inhibitory concentration method were used for testing. The extract was most effective against *Serratia rubidaea* CCM 4684 and *Saccharomyces cerevisiae* CCM 8191.

Janovská et al. (2003) demonstrated that extracts from *Tussilago farfara, Chelidonium majus,* and *Schisandra chinensis* had proven antimicrobial activity. Extract from each plant had significantly different activity against tested microorganisms, but various extracts showed greater antimicrobial activity against *Bacillus cereus* and *Staphylococcus aureus* than against *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*.

Also, **Kokoska et al. (2002)** reported that *Tussilago farfara* extract had an antimicrobial effect on *Bacillus cereus* (15.63 mg.mL^{-1}) and *Staphylococcus aureus* (62.50 mg.mL^{-1}) by MIC susceptibility test. The antimicrobial effects of the coltsfoot extract against *Escherichia coli* were not determined, although sensitivity of *E. coli* was observed in other medicinal plants.

According to two statistical processing of disc diffusion method **Hleba et al. (2013)** indicated that the *Tussilago farfara* extract reached size of inhibition zones 16.7 ±3.65 mm for ampicillin and chloramphenicol resistant *Escherichia coli* isolated from conventional cow breeding and 8.3 ±1.41 mm for antibiotics when we consider that *Escherichia coli* has been isolated from organic mare breeding. In contrast, the methanolic extract of *Aesculus hippocastanum* (diameter of inhibition zones 9 ±1.88 mm) had the least antimicrobial effect.

Milovanović et al. (2007) investigated the antimicrobial effects of horsetail (*Equisetum arvense*) extract, with inhibition zones size 12.1 ±0.5 mm.

Modarresi-**Chahardehi et al. (2012)** compared various types of extract preparation of *Urtica dioica* related to antimicrobial activity. Ethyl acetate, hexane and chloroform extracts displayed higher antimicrobial activity than the other extracts. The highest growth inhibition of ethyl acetate extract was observed against *Bacillus cereus*, methicillin resistant *Staphylococcus aureus,* and *Vibrio parahaemolyticus*. Phenols from *Urtica dioica* are one of the major groups associated with the inhibition of microbial infections in cancer **(Dar et al., 2012)**. Also, *Urtica dioica* is a rich source of phytochemicals, such as phenolic compounds and minerals, that can be used as a potential source of useful drugs **(Ahmed et al., 2012)**.

Radulović et al. (2006) examined essential oil from *Equisetum arvense.* The study showed significant antimicrobial properties against all tested strains. Diameter of inhibition zone ranged from 23 to 37 mm with the

highest inhibition zone against Gram-negative bacteria *Salmonella enteritidis* (35 mm) and *Klebsiella pneumoniae* (37 mm). Significant reduction of bacterial growth was demonstrated in medically important pathogens, such as *Staphylococcus aureus* (28 mm). The antimicrobial activity was greater or similar to conventional antibiotics. In addition, activity against fungi *Candida albicans* and *Aspergillus niger* was observed. The study also suggested that Gram-negative bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Salmonella enteritidis* were more susceptible to tested essential oils than Gram-positive *Staphylococcus aureus.* Exception was Gram-negative *Escherichia coli* which were the most resistant of all tested bacteria.

According to the MIC and MBC method used, the sage extract oil showed an interesting activity against Gram-positive pathogens. The most sensitive was *Staphylococcus epidermidis* (MIC = 12.5 $\mu g.mL^{-1}$), but the oil also showed very good activity against *Bacillus cereus* and *Bacillus subtilis* (MIC = 25 $\mu g.mL^{-1}$ for both). As regards Gram-negative bacteria, the sample showed significant efficacy only against *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumoniae*, which showed the lowest MIC values (50 $\mu g.mL^{-1}$), while being moderately active against *Proteus vulgaris* and *Salmonella enteritidis* (MIC = 100 $\mu g.mL^{-1}$) **(Tenore et al., 2011)**.

Results of **Mazandarani et al. (2013)** show that oil from *Achillea millefolium* extract may be an alternative to antibiotics in the control of some Gram-positive and Gram-negative pathogens. The antibacterial activity of some *Agrimonia eupatoria* extracts against pathogenic bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*) and their action on wound healing in rats has been confirmed. The presence of certain active substances in both aqueous and ethanol extracts has also been demonstrated, indicating that *Agrimonia eupatoria* may exhibit antimicrobial activity. The results of this study showed that the ethanol extract was more effective at inhibiting the bacteria tested than the aqueous extract. *Pseudomonas aeruginosa* was most resistant to the action of ethanol extract, while *Escherichia coli* with the highest zone of inhibition of 20 mm was the most susceptible. There was moderate activity against *Staphylococcus aureus* with 15 mm inhibition zone after application of ethanol extract (10 $mg.mL^{-1}$).

With regard to the antimicrobial activity of *Melissa officinalis* oil, **Romeo et al. (2008) and Hussain et al. (2011)** indicated its antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Bacillus pumilus, Pseudomonas aeruginosa, Salmonella Poona, Escherichia coli* and *Listeria innocua*. Antimicrobial activity (expressed as $\mu g.mL^{-1}$) from four ethanolic extracts of *Achillea millefolium, Agrimonia eupatoria, Melissa officinalis* and *Tilia platyphyllos* against various strains of Gram-positive and Gram-negative bacteria. It was found that more susceptible to *Agrimonia eupatoria* extract with a MIC50 value of 0.80 $\mu g.mL^{-1}$ of *Bacillus cereus, Lactobacillus brevis* was less susceptible to *Agrimonia eupatoria* with a MIC50 value of 1.48 $\mu g.mL^{-1}$.

The bacteria *Lactobacillus hilgardii, Enterococcus faecalis,* and *Escherichia coli* were less sensitive to *Agrimonia eupatoria* extract and the values were higher MIC50 (MIC 2.56 – 17.06 $\mu g.mL^{-1}$). *Lactobacillus brevis* was more sensitive to *Melissa officinalis* with a MIC50 of 6.39 $\mu g.mL^{-1}$. In addition, the activity of *Achillea millefolium* against Gram-positive and Gram-negative bacteria was contrary to previous reports that antibacterial activity was limited to Gram-positive bacteria from medicinal herbs **(Ghaima, 2013)**.

CONCLUSION

Medicinal plants have been used for treating various diseases due to their beneficial effects and sources of bioactive secondary metabolites for long times. In recent years, research has been increasingly investigating their antimicrobial activity against various pathogens. The emphasis should be placed on further research and monitoring of their effects. The plants that we used in the research showed an antimicrobial effect against Gram-positive and Gram-negative bacteria.

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ESTIMATION OF PHENOLIC COMPOUNDS CONTENT AND ANTIOXIDANT ACTIVITY OF LEAVES EXTRACTS OF SOME SELECTED NON-TRADITIONAL PLANTS

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ABSTRACT

The aim of the research is the determination of the total antioxidant activity and the content of phenolic compounds of the leaves of 12 species of non-traditional plants, namely, *Amelanchier alnifolia* (Nutt.) Nutt. ex M. Roem., *Aronia mitschurinii* A.K. Skvortsov & Maitul., *Castanea sativa* Mill., *Chaenomeles japonica* (Thunb.) Lindl., *Cornus mas* L., *Diospyros kaki* L., *Diospyros lotus* L., *Diospyros virginiana* L., *Lycium barbarum* L., *Lycium chinense* Mill., *Pseudocydonia sinensis* (Thouin) C.K. Schneid., *Ziziphus jujuba* Mill. Total phenolic content was evaluated using the Folin-Ciocalteu reagent assay. Antioxidant activity was measured using two different methods (DPPH – 2,2-diphenyl-1-picrylhydrazyl, MRAP – molybdenum reducing antioxidant power). Significant variability was observed in phenolic compounds content and total antioxidant activity. Total polyphenol content ranged from 38.02 (*Z. jujuba*) to 80.58 (*C. sativa*) mg GAE.g^{-1} DM, total flavonoid content from 22.47 (*P. sinensis*) to 54.61 (*L. barbarum*) mg QE.g^{-1} DM and phenolic acids content from 3.51 (*A. mitschurinii*) to 24.67 (*Ch. japonica*) mg CAE.g^{-1} DM. All tested samples exhibited DPPH• radical scavenging activities with values from 6.92 (*A. mitschurinii*) to 9.0 (*C. mas*) mg TEAC.g^{-1} DM. Antioxidant activity by molybdenum reducing antioxidant power method ranged from 109.43 (*A. mitschurinii*) to 322.95 (*C. mas*) mg TEAC.g^{-1} DM. Differences between the species of non-traditional plants were significant in all observed parameters. Obtained results of phytochemical composition demonstrated the possibility of leaves' use of non-traditional plants as sources of valuable bioactive compounds with health-promoting and disease-preventing properties.

Keywords: non-traditional plants; leaves; phenolic compounds; antioxidant activity

INTRODUCTION

Fruits constitute is a large group of functional food, whose consumption delivers several health benefits, but very limited studies about the leaves of plants especially lesser-known and non-traditional plants, among which fruit plants. Biological activity in plants present in leaves has attracted much attention to their beneficial health effects **(Yildirim, Oktay and Bülaloúlu, 2001; Olszewska, 2011; Amjad and Shafighi, 2012; Nam, Jang and Rhee, 2017; Yılmaz and Seyhan, 2017; Bhatt et al., 2018)**. Leaves of non-traditional plants are one of the promising sources of antioxidants **(Ipatova et al., 2003; Calliste et al., 2005; Sakanaka, Tachibana and Okada, 2005; Priya and Nethaji, 2015; Ferlemi and Lamari, 2016; Klymenko, Grygorieva and Brindza, 2017; Urbanaviciute et al., 2019)**. They can use in the tea production and may have potential health benefits as a therapeutic aid in many illnesses which could be attributed to their antifungal, anti-inflammatory, antimicrobial and antioxidant activities. For example, tea made up from leaves of the plant *Camellia sinensis* (L.) Kuntze, is the second most consumed beverage in the world **(Costa, Gouveia and Nobrega, 2002; Rietveld and Wiseman, 2003)**.

The leaves *Ziziphus jujuba* have been used for herbal tea as a folk medicine for hemorrhaging, diarrhea **(Mahajan and Chobda, 2009)** and also been used to improve sleep, nourish the heart and soothe the nerves **(Zhang et al., 2014)**. The effects of *Diospyros virginiana* leaf and bark were comparable to that of standard drug, Silymarin. The ethanolic extract *Diospyros virginiana* leaf and bark is not only an effective hepatoprotective agent but also possesses significant antioxidant activity **(Priya and Nethaji, 2015)**.

The leaves of *Diospyros kaki* are most widely used in countries in eastern Asia, including China, Korea, and Japan **(Ahn et al., 2017)**. They contain abundant bioactive chemicals, such as flavonoids, polyphenols, organic acids, and vitamins, which could contribute to their pharmacological characteristics, such as their potent radical-scavenging and antioxidant properties **(Kim et al., 2006; Lee et al., 2006)**. The leaves of *Diospyros kaki* show medicinal effects against hemostasis, diuresis, constipation, and hypertension, have beneficial effects on

eye diseases in humans **(Ryu et al., 2015; Xie et al., 2015; Ahn et al., 2017)**.

Aronia melanocarpa leaf extract effectively reduced lipid and protein peroxidation in brain homogenates obtained from rats subjected to immobilization-induced oxidative stress **(Cuvorova et al., 2005)**.

Scientific hypothesis

The results of this study will provide new knowledge and useful information about the content of phenolic compounds in some selected non-traditional plants leaves and the antioxidant activity of their extracts, which will give a wide range of possibilities to employing these plants as the sources of phenolic compounds.

MATERIAL AND METHODOLOGY

Selection of plants

Objects of this study were leaves of 12 species of non-traditional plants, namely, *Amelanchier alnifolia* (Nutt.) Nutt. ex M. Roem., *Aronia mitschurinii* A.K. Skvortsov & Maitul., *Castanea sativa* Mill., *Chaenomeles japonica* (Thunb.) Lindl., *Cornus mas* L., *Diospyros kaki* L., *Diospyros lotus* L., *Diospyros virginiana* L., *Lycium barbarum* L., *Lycium chinense* Mill., *Pseudocydonia sinensis* (Thouin) C.K. Schneid., *Ziziphus jujuba* Mill. (Figure 1). The raw materials were collected in the 2018 August on the experimental collection of the Institute of Biological Conservation and Biosafety, the Slovak University of Agricultural in Nitra.

The collected samples were packed in cotton bags and air-dried for several days. The air-dried samples were finely powdered using laboratory blender and kept in zip-locked bags until further analysis. Duplicate specimens were collected for the herbarium preparation.

Chemicals

All the chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and CentralChem (Slovakia).

Preparation of sample extracts

The dry non-traditional plants leaves were used for the detection of total phenolic content and total flavonoid content. An amount of 0.25 g of each sample was extracted with 20 mL of 80% ethanol for 24 h. Then, the sample in 80% ethanol was centrifuged at 4605 RCF (Rotofix 32 A, Hettich, Germany) for 10 min and the supernatant was used for measurement with the DPPH and molybdenum reducing antioxidant power methods.

Total polyphenol, flavonoid, and phenolic acid content

The total polyphenol content (TPC) was measured by the method of **Singleton and Rossi (1965)** using the Folin-Ciocalteu reagent. A quantity of 0.1 mL of each sample was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 min in darkness, the absorbance at 700 nm was measured with the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25 – 300 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard. The results were expressed in $mg.g^{-1}$ DM gallic acid equivalent.

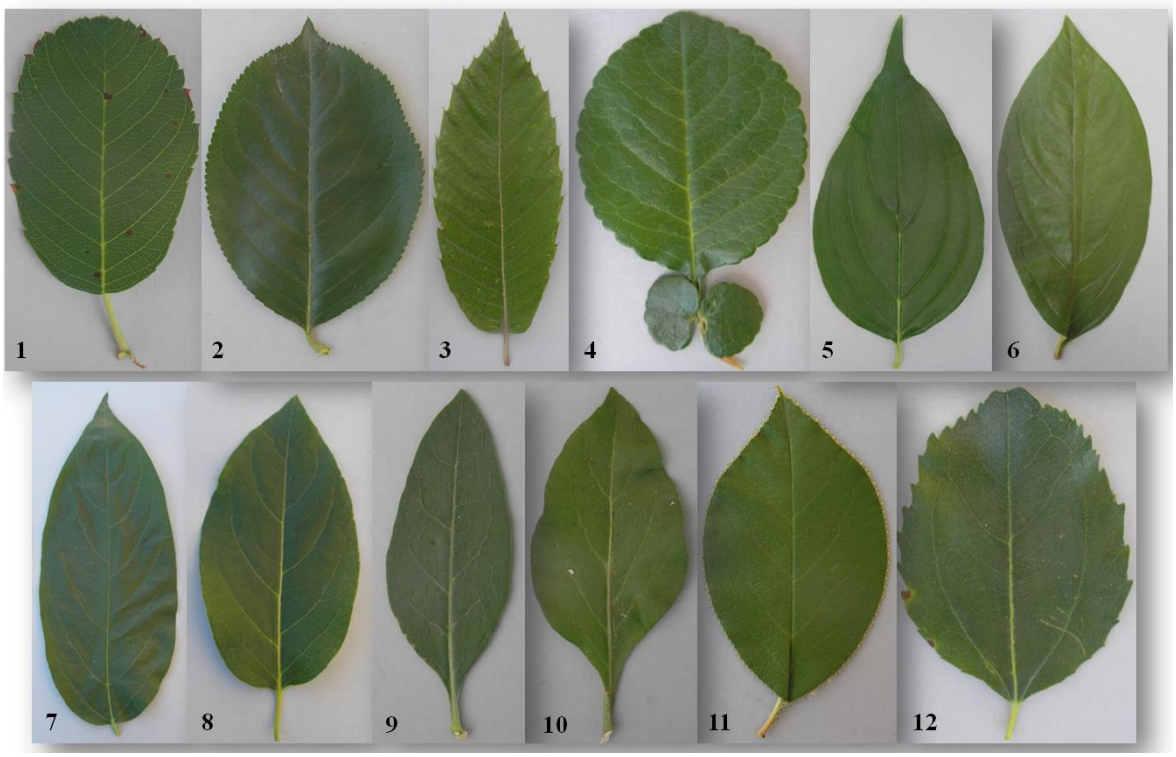

Figure 1 Leaves of non-traditional plants. Note: 1 – *Amelanchier alnifolia* (Nutt.) Nutt. ex M. Roem.; 2 – *Aronia mitschurinii* A.K. Skvortsov & Maitul.; 3 – *Castanea sativa* Mill.; 4 – *Chaenomeles japonica* (Thunb.) Lindl.; 5 – *Cornus mas* L.; 6 – *Diospyros kaki* L.; 7 – *Diospyros lotus* L.; 8 – *Diospyros virginiana* L.; 9 – *Lycium barbarum* L.; 10 – *Lycium chinense* Mill.; 11 – *Pseudocydonia sinensis* (Thouin) C.K. Schneid.; 12 – *Ziziphus jujuba* Mill.

The total flavonoid content (TFC) was determined by the modified method described by **Shafii et al. (2017)**. An aliquot of 0.5 mL of the sample was mixed with 0.1mL of 10% (w/v) ethanolic solution of aluminum chloride, 0.1 mL of 1 M potassium acetate and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (1 – 400 mg/L; R2 = 0.9977) was used as the standard. The results were expressed in $mg.g^{-1}$ DM quercetin equivalent.

Total phenolic acid (TPA) content was determined using the method of **Farmakopea Polska (1999)**. A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10% $NaNO_2$+10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1 – 200 mg/L, $R^2 = 0.999$) was used as a standard and the results were expressed in $mg.g^{-1}$ DM caffeic acid equivalents.

Determination of antioxidant activity

Free radical scavenging activity

Free radical scavenging activity of samples was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(Sanches-Moreno et al., 1998)**. An amount of 0.4mL of the sample was mixed with 3.6mL of DPPH solution (0.025 g DPPH in 100 mL methanol). The absorbance of the reaction mixture was determined with the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 $mg.L^{-1}$; $R^2 = 0.989$) was used as the standard and the results were expressed in $mg.g^{-1}$ DM Trolox equivalents.

Molybdenum reducing antioxidant power

Molybdenum reducing (MRP) antioxidant power of samples was determined by the method of **Prieto et al. (1999)** with slight modifications. The mixture of the sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90 °C for 120 min, then rapidly cooled. The absorbance at 700 nm was detected with the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10 – 1000 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard and the results were expressed in $mg.g^{-1}$ DMTrolox equivalent.

Statistical analysis

Basic statistical analyses were performed using PAST 2.17. Data were analyzed with ANOVA test and differences between means compared through the Tukey-Kramer test ($\alpha = 0.05$). The variability of all these parameters was evaluated using descriptive statistics. Correlation coefficients were calculated by CORR analysis.

RESULTS AND DISCUSSION

The main classes of natural antioxidant compounds such as flavonoids and phenolic acids often show a high correlation with antioxidant activity and have been identified in following non-traditional plants species: *Aronia melanocarpa* **(Tolić et al., 2015)**, *Cydonia oblonga* **(Bystrická et al., 2017)**, *Ziziphus jujuba* **(Ivanišová et al., 2017)**, *Diospyros virginiana* **(Grygorieva et al., 2018)**, *Sambucus nigra* **(Horčinová Sedláčková et al., 2018)**, *Asimina triloba* **(Brindza et al., 2019)**, *Cornus mas* **(Klymenko et al., 2019b)**, *Chaenomeles japonica* **(Klymenko et al., 2019a)** and other.

Total phenolic acid (TPA), flavonoids (TFC) and polyphenols (TPC) contents

Phenolic compounds have been proven to have a particularly strong antioxidant effect **(Scalbert et al., 2005; Pandey and Rizvi, 2009)**, which is closely related to the anti-inflammatory **(Pastore et al., 2009)**, strong antimicrobial **(Cushnie and Lamb, 2005)**, antiviral **(Chávez et al., 2006)**, and anticancer **(Kandaswami et al., 2005)** effect. Based on a large amount of scientific data proving the beneficial effect of phenolic compounds in humans, it is appropriate to perform estimation of these compounds content of leaves extracts of some selected non-traditional plants.

The amount of total phenolic acid content varied with the plant species (Figure 2). Total phenolic acids were ranged from 3.51 (*Aronia mitschurinii*) to 24.67 (*Chaenomeles japonica*) mg CAE.g^{-1} DM.

Comparing the works of literature, **Barreira et al. (2010)** reported a total phenol content of 228.37 – 522.98 $mg.g^{-1}$ of *Castanea sativa*. According to **Lavola, Karjalainen and Julkunen-Tiitto (2012)**, total phenolic acid content for *Amelanchier alnifolia* cultivars was from 22.78 to 26.75 $mg.g^{-1}$ DW. In this case, chlorogenic acids had maximal values (17.55 – 20.16 $mg.g^{-1}$). Also, this study showed that that among investigated organs of *A. alnifolia* cultivars leaves had the most content phenolic acids as well as other phenolic compounds. In our study leaves, extracts of this species showed less content of phenolic acids. The total phenolic contents of water extracts of *Cornus mas* was 341.09 $mg.g^{-1}$ **(Stankovic et al., 2014)**. Methanol extracts of *Ziziphus jujuba* had total phenolic content 68.10 $mg.g^{-1}$ **(Al-Saeedi et al., 2016)**. The total amount of phenolic compounds in the methanol extracts of *Chaenomeles japonica* leaves varied from 12.94 to 64.79 mg GAE.$100g^{-1}$ **(Urbanaviciute et al., 2019)**. The total phenolic contents have been investigated in other plant leaf extracts, including *Mangifera indica* L. (65 $mg.g^{-1}$), *Anacardium occidentale* L. (58.57 $mg.g^{-1}$), *Cymbopogon citratus* (DC.) Stapf (28.30 $mg.g^{-1}$), *Carica papaya* L. (21.80 $mg.g^{-1}$) **(Iyawe and Azih, 2011)**, *Euphorbia* spp. (19.10 – 20.30 $mg.g^{-1}$) **(Gapuz and Besagas, 2018)**, and *Azadirachta indica* A. Juss. (14.43 $mg.g^{-1}$) **(Iyawe and Azih, 2011)**.

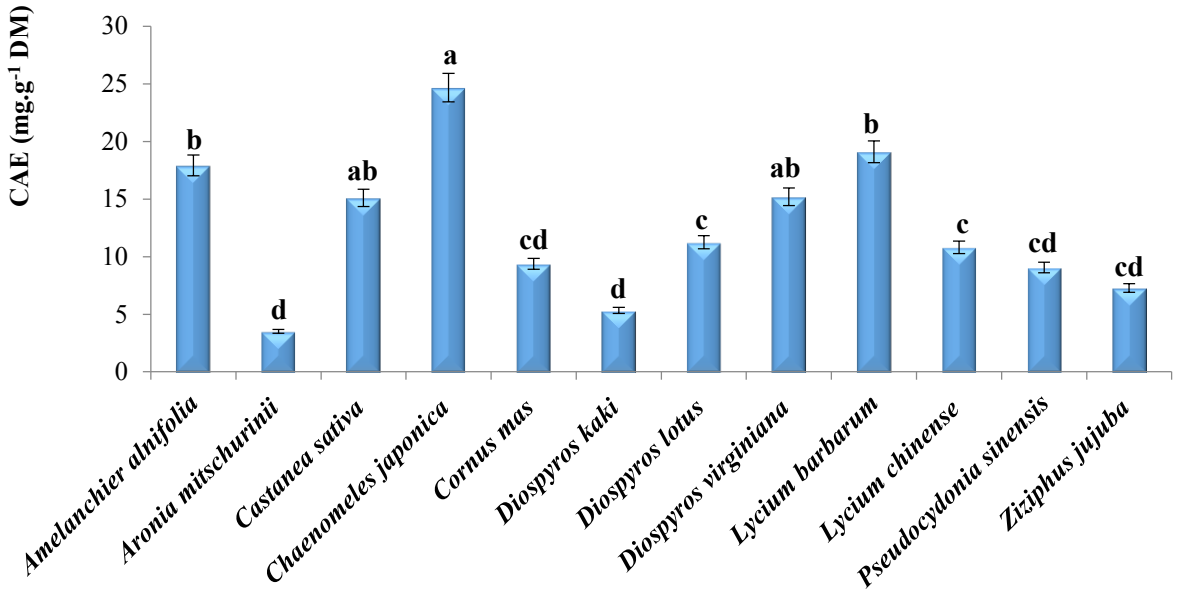

Figure 2 Total phenolic acid content in leaves of non-traditional plants.
Note: different superscripts in each column indicate the significant differences in the mean at p <0.05; CAE – caffeic acid equivalent.

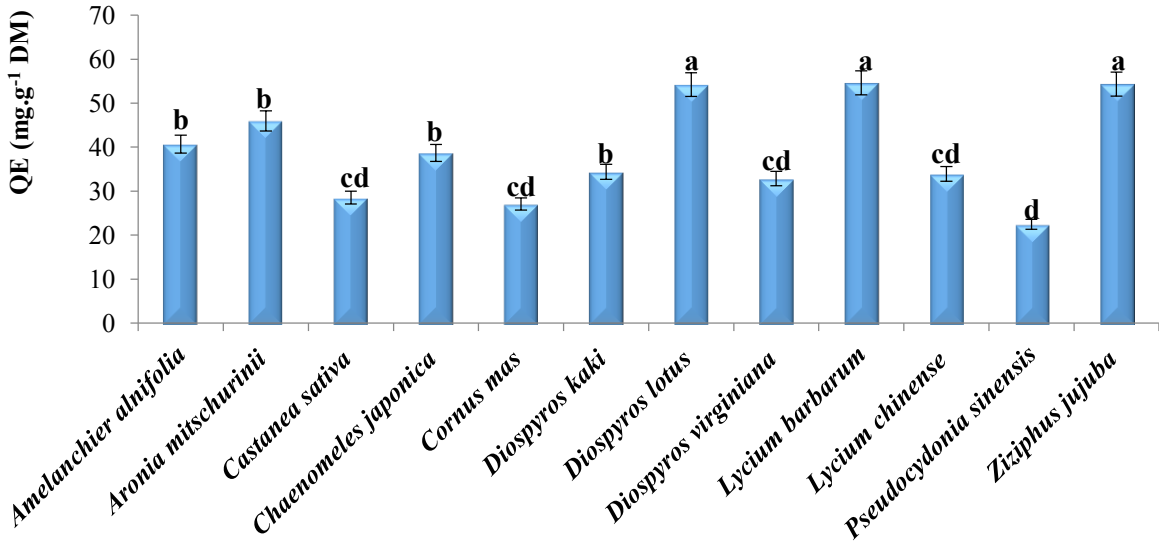

Figure 3 Total flavonoid content in leaves of non-traditional plants.
Note: different superscripts in each column indicate the significant differences in the mean at p <0.05); QE – quercetin equivalent.

The biological effects of many plant species depend on flavonoids; therefore, studies on the variation in their content are important and relevant **(Liaudanskas et al., 2014)**.

In the present work, *Lycium barbarum*, *Ziziphus jujuba* and *Diospyros lotus* leaf extract showed the highest amount of flavonoids (54, 61, 54.35 and 54.21 mg QE.g^{-1} DM, respectively) (Figure 3). The least value of flavonoids determined in extracts of *Pseudocydonia sinensis* (22.47 mg QE.g^{-1} DM).

According to **Barreira et al. (2010)**, total flavonoid contents *Castanea sativa* 73.31-90.39 mg.g^{-1}. Water extracts of *Cornus mas* leaves had total flavonoid contents 22.18 mg.g^{-1} **(Stankovic et al., 2014)**. *Aronia mitschurinii* leaves extracts in another study showed the content of flavonoids 103.6 – 163.7 mg CE.g^{-1} DW **(Thi and Hwang, 2014)**. Also, **Shahin et al. (2019)** found that the total content of flavonoids in dried leaves of *A. melanocarpa* was 96.16 mg.mL^{-1}. It should be noted that most studies about antioxidant parameters of *Aronia* species concerning of berries. The value of this parameter for *Ziziphus jujuba* methanol extracts was 90.28 mg.g^{-1} **(Al-Saeedi et al., 2016)** but ethanol extract of this species in our study was less. A study by **Aryal et al. (2019)** showed the flavonoid content in methanol leaves extracts eight selected wild vegetables from Nepal ranged from 37.86 to 66.1 mg QE.g^{-1}.

Natural plant antioxidants include main classes such as phenolic compounds, vitamins, carotenoids, etc. Phenolic compounds are a large group of antioxidants that can have structures from simple molecules to polyphenols (like flavonoids). Moreover, polyphenol compounds

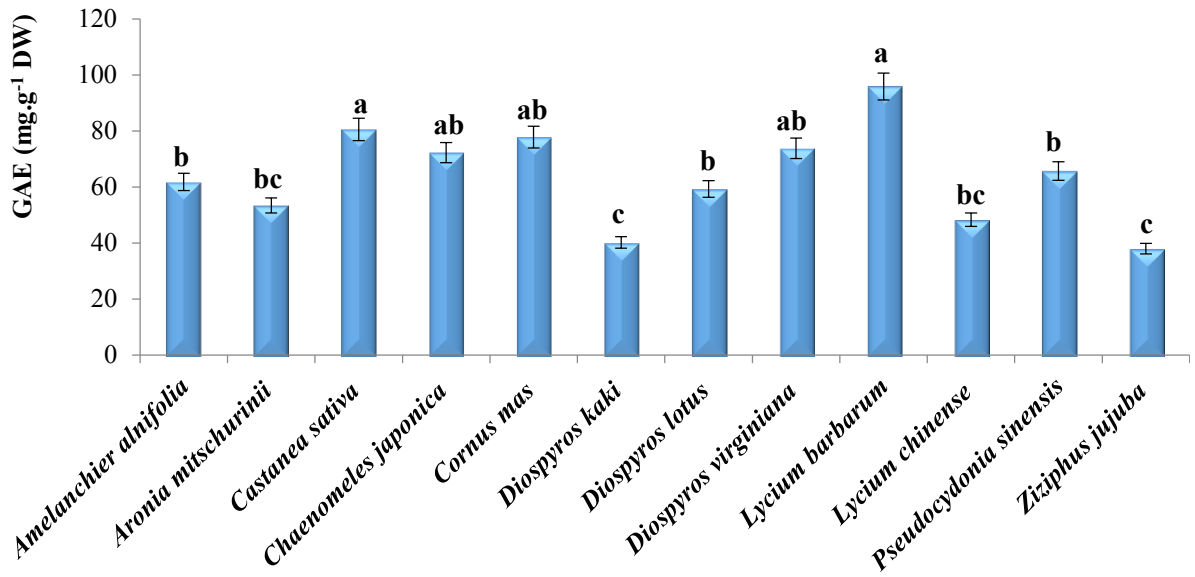

Figure 4 Total polyphenol content in leaves of non-traditional plants.
Note: different superscripts in each column indicate the significant differences in the mean at p <0.05; GAE – gallic acid equivalent.

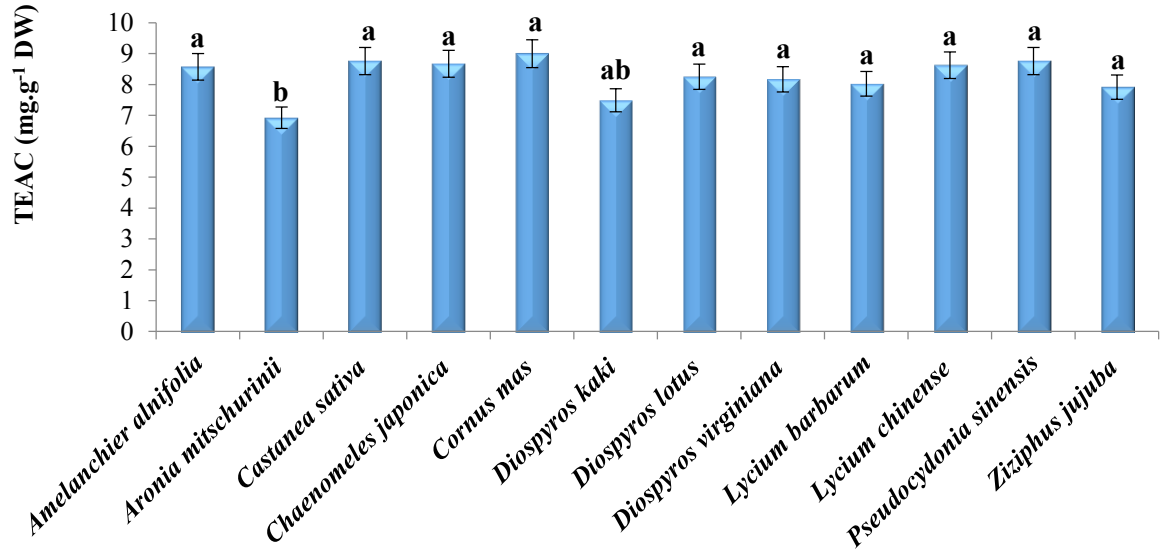

Figure 5 Antioxidant activity in leaves of non-traditional plants evaluated by the DPPH method.
Note: different superscripts in each column indicate the significant differences in the mean at p <0.05; TEAC – Trolox equivalent antioxidant capacity.

characterized by numerous activities that can be useful in human life such as antimicrobial, antifungal, anti-inflammatory, etc. **(Lourenço, Moldão-Martins and Alves, 2019)**.

The concentrations of total polyphenols content in non-traditional plant leaves are presented in Figure 4.

The highest total polyphenol content was obtained from *Lycium barbarum* and *Castanea sativa* (95.84 and 80.58 mg GAE.g^{-1} DW, respectively). In contrast, the lowest polyphenol content (40.24 and 38.02 mg GAE.g^{-1} DW, respectively) was obtained from *Diospyros kaki* and *Ziziphus jujuba* leaves.

Thi and Hwang (2014) found that *Aronia mitschurinii* total polyphenol content varied from 139.3 to 250.8 mg GAE.g^{-1} DW showed higher results than in the present study. The result of another study represented by **Shahin et al. (2019)** related to *Aronia melanocarpa* demonstrated that the total polyphenol content of dried leaves was 765.63 mg GAE.g^{-1}. As reported **Męczarska et al. (2017)**, leaves of *Amelanchier alnifolia* demonstrated the total content of polyphenols 185.23 mg GAE.g^{-1} DW that was 3 times less comparing with our result.

The antioxidative ability of leaves extracts

After studying the total phenolic compounds content it is important to examine and assess the antioxidant activity in the extracts of non-traditional plant species leaves. The results obtained during studies will be useful for the assessment and standardization of the quality of plant raw materials and will allow predicting an antioxidant effect of leaves non-traditional plants species.

The DPPH radical scavenging activities of non-traditional plant leaves at different stages of growth are shown in Figure 5. All tested samples exhibited DPPH•

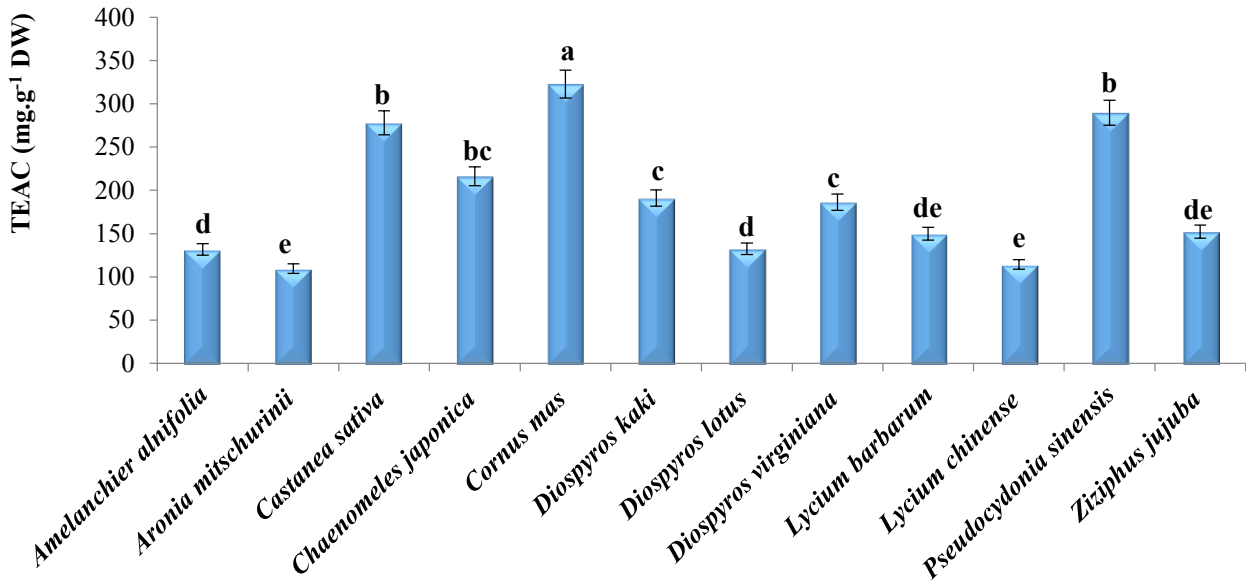

Figure 6 Antioxidant activity in leaves of non-traditional plants evaluated by the molybdenum reducing antioxidant power (different superscripts in each column indicate the significant differences in the mean at p <0.05); TEAC – Trolox equivalent antioxidant capacity.

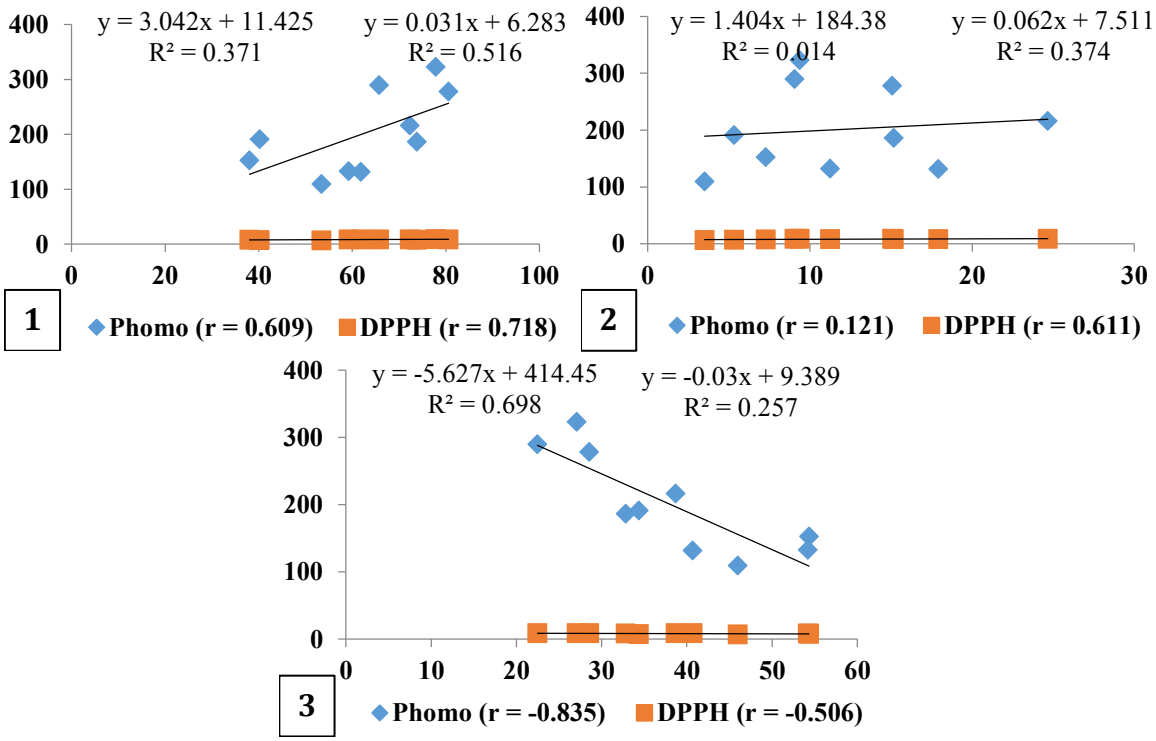

Figure 7 Correlation between antioxidant activity and TPC (1), TPA (2), and TFC (3) of non-traditional plants leaves extract.

radical scavenging activities with values from 6.92 (*Aronia mitschurinii*) to 9.0 (*Cornus mas*) mg TEAC.g^{-1} DW.

Serteser et al. (2009) reported that the methanolic extracts of the *Cornus mas* fruits showed EC50 (mg/ml) (DPPH reduction) values as 0.71.

The DPPH radical scavenging activity of the distilled water and 80% ethanol extracts of samples *Aronia mitschurinii* increased in a concentration-dependent manner (12.5~100 µg.mL^{-1}) **(Thi and Hwang, 2014)**. *Ziziphus spina-christi* (L.) Desf. ethanolic extracts exhibited good radical scavenging activity, with IC50 (the extract concentration providing 50% of inhibition) values of 54.3 µg.mL^{-1}, respectively (Khaleel et al., 2016). In the leaves of wild-growing plants of Nepal, the DPPH radical scavenging potency with a minimum IC50 value was recorded from 9.89 to 45.68 mg.mL^{-1} **(Aryal et al., 2019)**.

Antioxidant activity by molybdenum reducing antioxidant power method ranged from 109.43 (*Aronia mitschurinii*) to 322.95 (*Cornus mas*) mg TEAC.g^{-1} DM (Figure 6).

Comparison analyses of obtained data with results of other reviews often difficult to use in a similar study because of using different methods of determination of

antioxidant parameters, especially it is concerning standards that used and units.

The Pearson correlation coefficients between antioxidant activities and TPC, TPA, and TFC were depicted in Figure 7.

The result revealed the existence of a positive and negative correlation between tested antioxidant assays. TPC was significantly correlated with DPPH and phosphomolybdate assays (r = 0.609 – 0.718, p <0.05). The considerable correlation was noted in TPA and DPPH assay (r = 0.611).

CONCLUSION

In conclusion, the results of this study will provide new knowledge and useful information about the content of phenolic compounds in some selected non-traditional plants leaves and the antioxidant activity of their extracts, which will give a wide range of possibilities to employing these plants as the source of phenolic compounds. The highest total amounts of polyphenols and flavonoids were determined in the *Lycium barbarum* leaves (95.84 mg GAE.g^{-1} DW and 54.61 mg QE.g^{-1} DW, respectively). The preliminary experiments examining the antioxidant activity of leaf extracts of some selected non-traditional plants by the DPPH and phosphomolybdenum assays have shown that these extracts possess a strong antioxidant activity, which positively correlated with the total polyphenols content (r = 0.609-0.718, p <0.05). The ethanol extracts obtained from the apple leaves of the *Cornus mas* showed the highest TE values: 9.0 mg.g^{-1} DW by the DPPH method, 322.95 mg.g^{-1} DW by the molybdenum reducing antioxidant power. The findings of this study support the fact that leaves of some selected non-traditional plants are promising sources of potent antioxidants that can confirm the potential of investigated plants as a raw material in medical practice as well as the development and production of dietary supplements and cosmetic preparations rich in biologically active compounds.

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GENETIC DIVERGENCE IN TUNISIAN CASTOR BEAN GENOTYPES BASED ON TRAP MARKERS

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ABSTRACT

In the present study, the representatives of the genus *Ricinus communis* collected from 12 different parts of Tunisia were differentiated by the DNA fingerprinting patterns using 30 TRAP primers. The efficacy of the TRAP technique in this study is further supported by the obtained PIC values of the primers used in the analysis. PCR amplification of DNA using 30 primers for TRAP analysis produced 490 DNA fragments that could be scored in all 56 genotypes of Tunisian castor. The number of amplified fragments varied from 3 (TRAP 04 x arb 1, TRAP 22 x arb 3 and TRAP 23 x arb 3) to 13 (TRAP 56 x arb 2), and the amplicon size ranged from 100 to 1600 bp. Of the 490 amplified bands, 377 were polymorphic, with an average of 5.71 polymorphic bands per primer. To determine the level of polymorphism in the analysed group of Tunisian castor genotypes polymorphic information content (PIC) was calculated. The lowest values of polymorphic information content were recorded for TRAP 10 x arb 1 (0.555) and the highest PIC values were detected for TRAP 44 x arb 2 (0.961) with an average of 0.770. A dendrogram was constructed from a genetic distance matrix based on profiles of the 30 TRAP primers using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 56 Tunisian castor genotypes were clustered into five main clusters. Moreover, functional TRAP markers would be efficiently useful in genetic studies for castor genetic improvement.

Keywords: castor; DNA; dendrogram; PCR; PIC

INTRODUCTION

Castor bean (syn. castorbean, castor, castor-oil-plant), *Ricinus communis* L. (2n = 20, X = 10), is a species of flowering plant in the spurge family, *Euphorbiaceae*. It is an oilseed crop cultivated mainly in India, Mozambique, Brazil, and China **(FAOSTAT, 2014)**. The seeds of castor bean have around 35 – 55% oil, and the commercial standard is 44%. The oil percentage of the seeds varies depending on the cultivation environment and the cultivar **(Costa and Ramos, 2004)**. The hydroxylated fatty acid ricinoleic is approximately 80 – 90% of the total fatty acids, which gives castor bean oil (ricin oil) unique chemical and physical properties. Ricin oil is a renewable resource and raw material with various industrial applications (e.g., to manufacture paints, lubricants, cosmetics, pharmaceutical drugs, dyes, anilines, disinfectants, germicides, low-temperature lubricating oils, glues and adhesives, fungicide and insecticide bases, printing inks and varnishes, nylon and plastic), and more recently its use as biodiesel has been explored **(Mutlu and Meier, 2010)**.

Now that some of these lineages have been developed, there is a need to study the genetic divergence among them. The genetic divergence among genotypes of any species can be evaluated with molecular markers, for example, target region amplification polymorphism (TRAP) markers. TRAP markers are functional markers that allow combining fixed and specific primers with arbitrary primers **(Hu and Vick, 2003)**. These markers have high levels of polymorphism, which makes them a promising option for the genotypification of germplasm and identification of genes related to desirable agronomic characteristics. Besides, TRAP markers optimize the genetic gains in genetic improvement programs and are a valuable tool used by these programs to study genetic divergence **(Agarwal et al., 2008)**.

Genetic diversity in castor bean was assessed by using both dominant and codominant molecular markers (random amplified polymorphic DNA, RAPD) **(Reddy, Nadigatla and Mulpuri, 2015; Vivodík et al., 2015)**, inter-simple sequence repeats (ISSR) **(Wang et al., 2013; Vasconcelos et al., 2016)**, start codon targeted (SCoT) **(Kallamadi et al., 2015; Reddy, Nadigatla and Mulpuri,**

2015), amplified fragment length polymorphism (AFLP) **(Allan et al., 2008; Quintero et al., 2013)**, simple sequence repeat (SSR) **(Gálová et al., 2015; Rukhsar et al., 2017)**, expressed sequence tag-simple sequence repeats (EST-SSR) **(Kanti et al., 2015; Wang et al., 2017)**, and random microsatellite amplified polymorphic DNA (RMAPD) **(Dong et al., 2012)**, and also advanced molecular markers, such as single nucleotide polymorphism (SNP) **(Foster et al., 2010)**, sequencerelated amplification polymorphism (SRAP) **(Lu et al., 2010; Mei-Lian et al., 2012)** and methylation-sensitive amplification polymorphism (MSAP) **(He et al., 2017)**. The polymerase chain reaction (PCR) has been used by many authors, such as **Vyhnánek et al. (2015)**; **Bošeľová and Žiarovská (2016)**; **Ražná et al. (2016)**; **Žiarovská et al. (2017)**; **Simões et al. (2017b)**; **Žiarovská et al. (2018)**; **Ansari et al. (2018)**; **Balážová et al. (2018)**; **El-Fiki and Adly (2019)**; **Žiarovská et al. (2019)**; **Cehula et al. (2019)**; **Vivodík et al. (2019)**.

Scientific hypothesis

TRAP markers are polymorphic enough to distinguish individual genotypes of Tunisian castor germplasm.

MATERIAL AND METHODOLOGY

Fifty-six castor (*Ricinus communis* L.) genotypes were used in the present study. Seeds of castor were obtained from the University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia. The ricin genotypes were obtained from 12 regions of Tunisia: S- Souassi (5 genotypes), BT- Bouthay (4 genotypes), GH- Ghomrassen (5 genotypes), BA- Sidi bou ali (5 genotypes), MT- Matmata (4 genotypes), AG- Mateur (5 genotypes), N- Nefza (4 genotypes), MD- Mednine (5 genotypes), M- Mornag (5 genotypes), G- Gabes (4 genotypes), K- Kebili (5 genotypes), KJ- Ksar jedid (5 genotypes). Genomic DNA of castor cultivars was extracted from leaves of 14-day old plantlets with GeneJET Plant Genomic DNA Purification Mini Kit according to the manufacturer's instructions. DNA concentrations were estimated by UV-Vis spectrophotometer Q5000, Quawell.

Amplification of TRAP fragments was performed according to **(Simões et al., 2017a)** using decamer arbitrary primers (Table 1 and Table 2). Amplifications were performed in a 15 μL reaction volume containing 1.5 μL of DNA, 7.5 μL of Master Mix (Genei, Bangalore, India), 1.5 μL of primer, and 4.5 μL H_2O. Amplification was performed in a programmed thermocycler (Biometra, Germany) with the following cycle: 94 °C for 2 min; 5 cycles at 94 °C for 45 s, 35 °C for 45 s and 72 °C for 1 min; followed by 30 cycles at 94 °C for 45 s, 40 °C for 45 s, 72 °C for 1 min; and a final extension of 72 °C for 7 min. Amplified products were electrophoresed in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system.

Statistical analysis

A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the SPSS professional statistics version 17 software package was constructed. For the assessment of the polymorphism between genotypes ricin and usability RAPD markers in their differentiation, we used polymorphic information content (PIC) **(Weber, 1990)**.

RESULTS AND DISCUSSION

In the present study, the representatives of the genus *Ricinus communis* collected from 12 different parts of Tunisia were differentiated by the DNA fingerprinting patterns using 30 TRAP primers. The efficacy of the TRAP technique in this study is further supported by the obtained PIC values of the primers used in the analysis. PCR amplification of DNA using 30 primers (Table 1 and Table 2) for TRAP analysis produced 490 DNA fragments that could be scored in all 56 genotypes of Tunisian castor (Figure 2 and Table 3). The number of amplified fragments varied from 3 (TRAP 04 x arb 1, TRAP 22 x arb 3 and TRAP 23 x arb 3) to 13 (TRAP 56 x arb 2), and the amplicon size ranged from 100 to 1600 bp. Of the 490 amplified bands, 377 were polymorphic, with an average of 5.71 polymorphic bands per primer. Results indicated the presence of wide genetic variability among different genotypes of Tunisian castor. To determine the level of polymorphism in the analysed group of Tunisian castor genotypes polymorphic information content (PIC) was calculated. The lowest values of polymorphic information content were recorded for TRAP 10 x arb 1 (0.555) and the highest PIC values were detected for TRAP 44 x arb 2 (0.961) with an average of 0.770.

A dendrogram was constructed from a genetic distance matrix based on profiles of the 30 TRAP primers using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 56 Tunisian castor genotypes were clustered into five main clusters (Figure 1). Cluster 1 contained 2 unique genotypes of Tunisian castor (K-4 and BA-5) from different regions of Tunisia and cluster 2 was divided into two subclusters (2a and 2b). Subcluster 2a contained 7 genotypes of castor and subcluster 2b contained 4 genotypes of Tunisian castor. Cluster 3 contained 6 genotypes of Tunisian castor and cluster 4 was divided into 3 subclusters (4a, 4b, and 4c). Subcluster 4a contained 2 genotypes of Tunisian castor and subcluster 4b contained 6 genotypes of Tunisian castor and subcluster 4c contained 29 genotypes of Tunisian castor. (Figure 1).

TRAP markers were used by other authors **(Miklas et al., 2006; Hu et al., 2007; Yu et al., 2007; Alwala et al., 2008; Kwon et al., 2010; Yue et al., 2010; Andru et al., 2011; Barakat et al., 2013; Cheng et al., 2013; Crotti-Franco et al., 2014; Kumar et al., 2014; Carmo et al., 2015; Feng et al., 2015; Luo et al., 2015; Dias Kanthack Junior et al., 2020)**.

Table 1 Characterization of the fixed primers (target region amplification polymorphisms, TRAPs) used to genotype 56 lineages of Tunisian castor bean.

	TRAP Primer	Sequence (5' – 3')
1.	TRAP 01	CCACATCCAGCACCTTTTG
2.	TRAP 02	TGTGGAGCGTTGAGGATTC
3.	TRAP 03	TGCTCGCAGGCAAAGATAC
4.	TRAP 04	TGTCCCATATTTGCCAACG
5.	TRAP 15	CCGTGATTCTGGTGGTGAG
6.	TRAP 16	TTACAACTGCGGCATCTCC
7.	TRAP 10	CGGGTGGCATCAGTTACAG
8.	TRAP 11	GGCGGATGCTATCTGTGAA
9.	TRAP 22	CACTCGCCTGTTCAGCACT
10.	TRAP 23	AGCAAGCCGCACCTAAGAT
11.	TRAP 24	GTCCAAGCAAAAGCCACCT
12.	TRAP 25	CCACCAATCCAACGCATAG
13.	TRAP 19	AATGCCAGCACCTACACCA
14.	TRAP 30	CTTCTCAGTTGCCCGTTCA
15.	TRAP 31	CCACCAATGAACCAACTGC
16.	TRAP 32	TGCCGACTTCTCCTTTCCT
17.	TRAP 35	CCTCATCATCGTTGCTGCT
18.	TRAP 27	CGAAATCCTCCTGCTCCTC
19.	TRAP 28	GCCACCATCTTCACCACAG
20.	TRAP 37	GCTCACGCACTGGACTCAT
21.	TRAP 39	GCACCCGAAATCTTCCACT
22.	TRAP 40	CCACTCAACACCGTTCCAC
23.	TRAP 44	CGTCCACCCACACTTTCAC
24.	TRAP 46	CCAGTCACCGTTTGTTGCT
25.	TRAP 49	TCCTGTCCAATGCTGAACC
26.	TRAP 51	CCACCGAGAGAGCATACCA
27.	TRAP 52	GTGGCAAATGCTCACAGGT
28.	TRAP 53	TACAACTTCGGGTGGTGGA
29.	TRAP 55	TGATGGAAACCCTTGTGGA
30.	TRAP 56	CTTGTGCCCTACCAACTGC

Table 2 Arbitrary primers used to genotype the 56 lineages of Tunisian castor bean.

	Arbitrary primers	Nucleotide sequence (3' – 5')
1.	arb 1	GACTGCGTACGAATTGAC
2.	arb 2	GACTGCGTACGAATTTGA
3.	arb 3	GACTGCGTACGAATTGCA
4.	arb 4	GACTGCGTACGAATTAATT
5.	arb 5	GACTGCGTACGAATTTGCC
6.	arb 6	GACTGCGTACGAATTGACC

Zhang et al. (2013) assessment the genetic diversity and variation of *Pinellia ternata* collected from 43 populations in China using SRAP þ TRAP markers. A total of 13 SRAP primers in addition to 3 TRAP primer combinations yielded 292 bands in a total of which 286 were polymorphic (98.0%), with an average of 16 for each. The PIC value ranged from 0.88 to 0.95, with a mean polymorphic information content (PIC) of 0.92 over all the primers. **Luo et al. (2013)** developed and characterized sequence tags (ESTs)-simple sequence repeats (SSRs) and targeted region amplified polymorphism (TRAP) markers to examine genetic relationships in the persimmon genus *Diospyros gene* pool. In total, we characterized 14 EST-SSR primer pairs and 36 TRAP primer combinations, which were amplified across 20 germplasms of 4 species in the genus *Diospyros*. **Liu et al. (2016)** study the genetic structure and genetic diversity among and within the 21 populations using target region amplified polymorphism (TRAP) and simple sequence repeat (SSR) markers. Sixteen pairs of TRAP primers generated a total of 398 fragments, of which 396 (99.50%) were polymorphic; fourteen pairs of SSR primers generated a total of 60 fragments, of which 59 (98.33%) were polymorphic. **Al-Murish et al. (2013)** study efficiency of SRAP, TRAP, and SSR primers in detecting genetic variation among 17 *C. arabica* genotypes collected from the different valleys of Yafea City, Yemen, and estimate genetic similarity coefficients among these genotypes and classify them according to genetic relationships. The results of the present study demonstrated the presence of genetic variation among coffee genotypes within and between valleys.

In this study, **Liu et al. (2015)** estimated the genetic relationships within *P. aibuhitensis* using Target Region Amplified Polymorphisms (TRAP) and Amplified Fragment Length Polymorphisms (AFLP) that were derived from related populations on the coasts of China.

Table 3 Combinations of target region amplification polymorphism (TRAP) primers selected to analyze the polymorphism in 56 lineages of Tunisian castor bean.

Combinations	Total fragments	Polymorphism fragments	PIC
TRAP 01 x arb 1	8	6	0.896
TRAP 02 x arb 1	10	8	0.689
TRAP 03 x arb 1	6	6	0.874
TRAP 04 x arb 1	3	2	0.755
TRAP 15 x arb 1	8	8	0.854
TRAP 16 x arb 1	9	6	0.668
TRAP 10 x arb 1	11	5	0.555
TRAP 11 x arb 1	4	4	0.899
TRAP 22 x arb 1	5	4	0.877
TRAP 23 x arb 1	8	7	0.788
TRAP 24 x arb 1	12	8	0.786
TRAP 25 x arb 1	6	5	0.854
TRAP 19 x arb 1	5	5	0.869
TRAP 30 x arb 1	9	6	0.789
TRAP 31 x arb 1	9	5	0.745
TRAP 32 x arb 2	7	4	0.658
TRAP 35 x arb 2	7	7	0.780
TRAP 27 x arb 2	4	3	0.754
TRAP 28 x arb 2	8	6	0.666
TRAP 37 x arb 2	11	7	0.731
TRAP 39 x arb 2	12	9	0.591
TRAP 40 x arb 2	6	6	0.781
TRAP 44 x arb 2	5	4	0.961
TRAP 46 x arb 2	9	8	0.812
TRAP 49 x arb 2	8	8	0.739
TRAP 51 x arb 2	9	6	0.630
TRAP 52 x arb 2	4	3	0.891
TRAP 53 x arb 2	6	5	0.709
TRAP 55 x arb 2	12	11	0.900
TRAP 56 x arb 2	13	10	0.810
TRAP 01 x arb 3	11	9	0.712
TRAP 02 x arb 3	9	9	0.890
TRAP 03 x arb 3	6	5	0.731
TRAP 04 x arb 3	8	8	0.912
TRAP 15 x arb 3	7	4	0.611
TRAP 16 x arb 3	8	6	0.723
TRAP 10 x arb 3	9	5	0.600
TRAP 11 x arb 3	6	4	0.599
TRAP 22 x arb 3	3	3	0.896
TRAP 23 x arb 3	3	3	0.879
TRAP 24 x arb 3	8	8	0.911
TRAP 25 x arb 3	4	3	0.823
TRAP 19 x arb 3	4	4	0.781
TRAP 30 x arb 3	5	5	0.910
TRAP 31 x arb 3	5	5	0.901
TRAP 32 x arb 4	4	3	0.801
TRAP 35 x arb 4	6	5	0.780
TRAP 27 x arb 4	8	6	0.699
TRAP 28 x arb 4	9	6	0.689
TRAP 37 x arb 4	7	4	0.609
TRAP 39 x arb 4	7	7	0.839
TRAP 40 x arb 4	7	7	0.798
TRAP 44 x arb 4	8	5	0.698
TRAP 46 x arb 4	11	8	0.801
TRAP 49 x arb 4	10	6	0.639
TRAP 51 x arb 4	11	7	0.709
TRAP 52 x arb 4	9	6	0.806
TRAP 53 x arb 4	6	6	0.809
TRAP 55 x arb 4	5	5	0.796
TRAP 56 x arb 4	4	3	0.908
TRAP 01 x arb 5	4	3	0.869
TRAP 02 x arb 5	8	5	0.703
TRAP 03 x arb 5	7	6	0.666
TRAP 04 x arb 6	9	5	0.599
TRAP 15 x arb 6	10	5	0.669
TRAP 16 x arb 6	10	6	0.759
Averages	7.42	5.71	0.770

Note: PIC = polymorphism information content

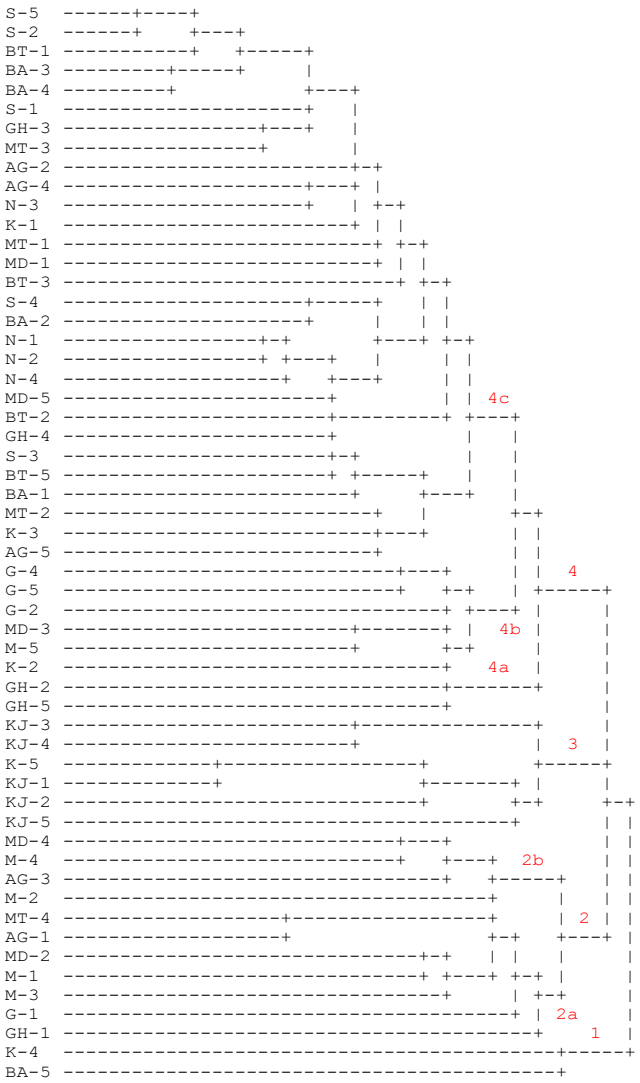

Figure 1 Dendrogram of 56 Tunisian castor genotypes prepared based on 30 TRAP markers.
Note: S – Souassi (5 genotypes), BT – Bouthay (4 genotypes), GH – Ghomrassen (5 genotypes), BA – Sidi bou ali (5 genotypes), MT – Matmata (4 genotypes), AG – Mateur (5 genotypes), N – Nefza (4 genotypes), MD – Mednine (5 genotypes), M – Mornag (5 genotypes), G – Gabes (4 genotypes), K – Kebili (5 genotypes), KJ – Ksar jedid (5 genotypes).

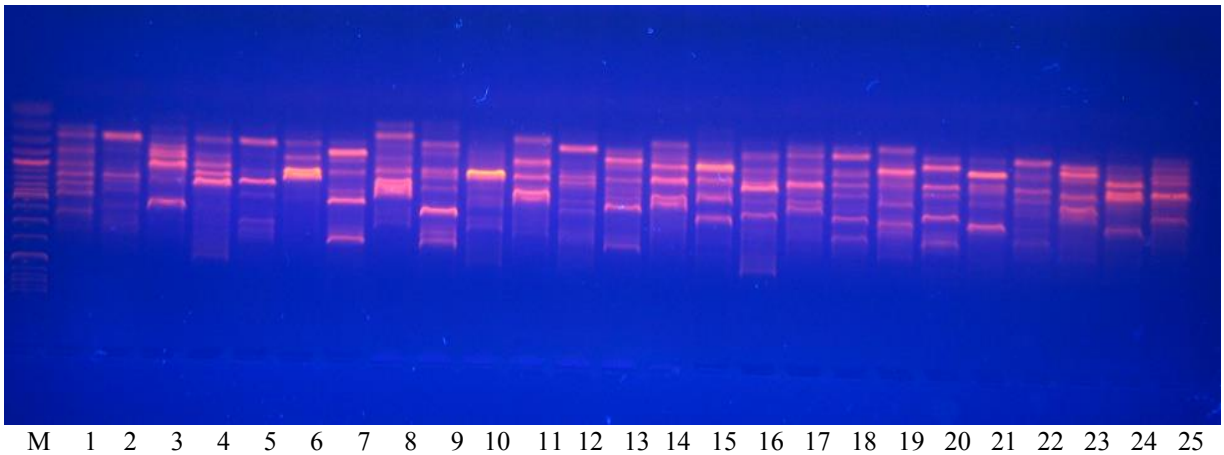

Figure 2 Electrophoretic profile, on 2% agarose gel, obtained from the amplification of the genomic DNA of 25 lineages of *Ricinus communis* L.
Note: (lanes 1-25) using TRAP 01 x arb 3 primer. Lane M: 100-bp molecular weight marker.

Among the TRAPs, 449 bands were observed in total, 439 of which (97.77%) were polymorphic between the *P. aibuhitensis* populations and were shared between at least four individuals. The Qinzhou (QZ) population had the highest PPB (78.62%), and the Dalian (DL) population had the lowest (60.13%). The objective of study **Farias da Silva et al. (2016)** was to analyze the genetic diversity of the clonal germplasm of the guarana plant using Target Region Amplification Polymorphism (TRAP) and Sequence-Related Amplification Polymorphism (SRAP) markers. Sixty clones of the guarana plant were analyzed; 18 were cultivars, eight were similar clones according to morpho-agronomic traits, and 34 were clones of a different origin. **Singh et al. (2017)** study the genetic variations among the twenty-five sugarcane genotypes employing functional molecular (TRAP) markers. Genetic diversity exists among sugarcane germplasm was exploited to identify promising genotypes bearing enviable agronomic traits (sucrose content and multiple disease resistance). Genetically diversified genotype could be exploited as proven parents in sugarcane hybridization programs to establish a promising cross. **Mirajkar et al. (2017)** study molecular marker profile using 57 markers, comprising of 27 TRAP and 30 SRAP markers in the gamma ray-induced sugarcane mutants. Collectively these markers produced 260 PCR amplicons among which 147 were polymorphic (56.54%). The TRAP marker-based analysis showed that the mutants AKTS-01 and AKTS-16 were more diverse (GS = 94 and 92%, respectively) than the rest of the mutants. In the study of **Fabriki-Ourang and Yousefi-Azarkhanian (2018)** target region amplification polymorphism (TRAP) and conserved region amplification polymorphism (CoRAP) markers were used for genetic diversity and relationship analysis of 25 Salvia ecotypes/species. Twelve TRAP and CoRAP primer combinations (four arbitrary primers and three fixed primers from *Salvia miltiorrhiza* expressed sequence tag sequences) amplified 180 loci, of which all were polymorphic. **Srivong et al. (2019)** study 17 sugarcane genotypes from Hawaii and Thailand using 12 target region amplification polymorphism (TRAP) markers and partial Sai nucleotide polymorphism. A total of 275 fragments were produced, of which 273 (99.27%) were polymorphic. The polymorphic information content (PIC) ranged from 0.912 – 0.959 with an average value of 0.938. Genetic similarity (GS) by Dice's similarity coefficient ranged from 0.19 – 0.81 with a mean of 0.44.

CONCLUSION

PCR amplification of DNA using 30 primers for TRAP analysis produced 490 DNA fragments that could be scored in all 56 genotypes of Tunisian castor. The number of amplified fragments varied from 3 (TRAP 04 x arb 1, TRAP 22 x arb 3 and TRAP 23 x arb 3) to 13 (TRAP 56 x arb 2), and the amplicon size ranged from 100 to 1600 bp. Of the 490 amplified bands, 377 were polymorphic, with an average of 5.71 polymorphic bands per primer. To determine the level of polymorphism in the analysed group of Tunisian castor genotypes polymorphic information content (PIC) was calculated. A dendrogram was constructed from a genetic distance matrix based on profiles of the 30 TRAP primers using the unweighted pair-group method with the arithmetic average (UPGMA). TRAP markers could be used to select elite parent genotypes, analysing genetic variation, utilization of genotype potential for trait improvement for adaptation to stress environment. It is therefore suggested that a focused breeding scheme should be adopted while analyzing genome diversity for parent selection to gain maximum value and practical impact on breeding program. TRAP markers exhibited remarkable discriminatory power for genetic diversity analysis.

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EFFECT OF SUPPLEMENTATION WITH SOLID-STATE FERMENTED FEED IN THE DIET OF LAYING HENS ON EGG QUALITATIVE VARIABLES

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ABSTRACT

The aim of this experiment was to evaluate the effect of the supplementation of laying hens diet with solid-state fermented feed on egg qualitative variables. The diet of laying hens was supplemented with 10% and 15% of solid-state feed fermented by the low filamentous fungal strain *Mortierella alpina* CCF 2861. For the trial, 30 Lohmann Brown classic layers, aged 17 weeks, were selected and individually weighed and divided into three groups (control and two experimental groups). The control group of laying hens was fed with basic feed mixture and the experimental groups received the same diet as a control group, but enriched with supplementation of solid-state fermented feed. The first experimental group was fed a diet supplemented with 10% of fermented feed and the second experimental group with 15% supplementation. The following egg qualitative variables were observed: the egg weight, Haugh units, quality grade, air cell depth, percentage of the shell, yolk and albumen, eggshell breaking force, pH of egg yolk and albumen, egg yolk colour, and antioxidant activity with the extent of lipid oxidation in egg yolk samples. The pH of yolk and albumen did not show differences between all examined eggs originating from the experimental groups of laying hens (p >0.05). The eggs from both experimental groups had a significantly higher eggshell hardness than eggs produced by the hens of the control group (p <0.05). Antioxidant activity of egg yolk of experimental samples increased with the supplementation of fermented feed in the diet of laying hens (p <0.05). The specific lightness of egg yolk colour increased significantly in the experimental group with 15% of supplementation (p <0.01). The obtained results showed that feeding laying hens with fermented feed positively affected the quality of produced eggs. This was the first study and further investigation before using the fermented feed in commercial laying hen farms is necessary.

Keywords: laying hen; egg; quality; solid-state fermented feed; colourimetry

INTRODUCTION

Eggs are an important source of polyunsaturated fatty acids (PUFA) in human nutrition. PUFA are biologically active substances that are beneficial for human health. The positive effect of PUFA on health is very well known. They are components of cell membranes and precursors of eicosanoids **(Gładkowski et al., 2011)**. They regulate architecture, dynamics, phase transition, and permeability of membranes as well as the behaviour of some membrane-bound proteins. In addition, PUFA, as essential compounds, are precursors of a multitude of diverse metabolites, such as prostaglandins, leukotrienes, and hydroxy fatty acids **(Ghadiri et al., 2016; Wang et al., 2017)**. Eggs are also interesting from the viewpoint that the content of individual significant n-3 and n-6 PUFAs in the lipid portions of an egg can be very easily changed by the composition of the fatty acids in the feed of laying hens. The weight and composition of a table egg are dependent on heredity, age, season, diet, and other factors **(Kusum et al. 2018)**. The main chemical components of hen egg are 12% lipids, 12% proteins, and the rest is water and small amounts of carbohydrates and minerals **(Sugino, Nitoda and Juneja, 1997)**. Most of the proteins are present in the egg white and the egg yolk, amounting to 50% and 44%, respectively; the eggshell contains the rest of the proteins **(Kusum et al. 2018)**. The protein fraction is distributed in both egg white (ovalbumin, ovotransferrin, ovomucoid, ovomucin, etc.) and yolk (high-density lipoproteins, low-density lipoproteins and livetins) **(Nimalaratne and Wu, 2015)**. The yolk accounts for slightly over one-third of the edible portion, but it yields three-fourths of the calories and provides all or most of the fat in whole eggs The yolk comprises 48% water, 16% protein, 32.6% fat, and some minerals and vitamins. The white consists of 88% water, 10% protein, and some minerals **(Ren, Wu and Renema, 2010)**. The yolk is a complex milieu containing 68% low-density lipoproteins (LDL), 16% high-density lipoproteins (HDLs), 10% livetins and other soluble proteins, and 4% phosvitins **(Réhault-Godbert, Guyot and Nys, 2019)**. The amount of lipid in the egg white is negligible (0.01%) compared with the amount present in the yolk. The shell makes up 11% of the weight of an egg, and approximately 98% of the shell consists of calcium. Carbohydrates are a minor

component of hen eggs. Their average content is about 0.5 g per egg, 40% of which is present in the yolk. **(Ren, Wu and Renema, 2010; Kusum et al. 2018)**.

Many egg proteins such as ovalbumin, ovotransferrin, phosvitin, egg lipids such as phospholipids, as well as certain micronutrients such as vitamin E, vitamin A, selenium, and carotenoids, are reported to have antioxidant properties, which prevents or removes oxidative damage to a target molecule by the regulation of antioxidant defence or inhibition of radicals production **(Nimalaratne and Wu, 2015)**.

Numerous studies have been published on the supplementation of hen's diets with ALA-rich seeds or oil; EPA/DHA-rich fish oil, fish oils combined with humic preparations **(Gładkowski et al., 2011),** microalgae (such as biomass of *Spirulina maxima*) **(Saeid and Chojnacka, 2015; Neijat, et al., 2016; Saeid et al., 2016)**.

Industrial processing of feeds destined for animal consumption and human nutrition results in high amounts of agroindustrial residues. Most of these residues have nutritional potential **(Graminha et al., 2008)**. These residues have been classified as agro-industrial by-products and recently they have been receiving greater attention **(Eun et al., 2006)**. In terms of cost efficiencies, the replacement of expensive conventional feedstuffs in animal diets with cheaper unconventional fermented feedstuffs may further encourage the use of the latter **(Sugiharto and Ranjitkar, 2019)**.

Solid-state fermentation (SSF) is the oldest known fermentation technique, which imitates the natural environment of the microorganism **(Marcinčák et al., 2018)**. SSF is characterized as a process in which microorganisms grow on a moist solid substrate in the absence of free water, simulating the fermentation reactions occurring in nature **(Pandey, 2003)**. Low filamentous fungi strains used in the SSF process simultaneously decrease the anti-nutrient compounds in the substrates and partially hydrolyse substrate biopolymers, the pre-fermented mass with a high content of PUFA, which may be used as an inexpensive food and feed supplement **(Čertík et al., 2008)**.

Several strains of oleaginous lower fibrous fungi, in particular Mortierella, Cunninghamella, Mucor, Thamnidium, Pythium and Thraustochytrium, are a good source of PUFAs **(Čertík et al., 2013)**. *Mortierella alpina* is a food-grade oleaginous fungus with the ability to release a high level of PUFAs, especially arachidonic acid (C 20:4-n-6) and eicosapentaenoic acid (C 20:5-n-3) **(Dai et al., 2016)**. The solid-state fermented feed, prepared by fermentation of distiller's dried grains with solubles and soybean meal by *Mortierella alpina* respectively, was successfully applied in poultry nutrition, resulting in an enhanced amount of PUFA in chicken breast meat **(Yang and Zhang, 2016)**. The product from the SSF process increased the PUFA content in chicken breasts and enhanced the proportions of n-6 and n-3. Hence, the requirement for new natural nutritional products could be potentially fulfilled by the method of the fermentation process **(Klempová et al. 2013)** and the subsequent use of the fermented products in the diet of laying hens. Pertaining to our studies, it should improve the egg quality and nutritional value of produced eggs, e.g. by the increase of PUFA content in egg yolk.

One of the most important factors is feeding of the laying hens, not only because of the effect on egg yolk colour but also because of the quality and safety of the final product. Many external and internal factors **(Tůmová and Ebeid, 2005; Dvořák et al., 2007)** affect the quality of eggs used in human nutrition. A multitude of scientific literature and data exist that discuss the replacement of corn, which represents the main ingredient of poultry diets, accounting for 60-70% of feed costs **(Laganá et al., 2011)**. For this purpose, soybean meal, sorghum, broken rice, millet, cassava meat, etc. have been proposed. However, a carotenoid source, or annatto (*Bixa Orellana* L.), and curcumin (main pigment in turmeric roots – *Turmeric longa* L.), must be added to ensure egg yolk pigmentation **(Assuena et al., 2008**. On the other hand, the dietary carrot can influence both the physical characteristics of the egg and yolk colour, the latter of which contains a higher amount of carotenes and a lower amount of xanthophylls and paprika **(Spasewski et al., 2018)**. It is known that egg yolk colour is affected mostly by the diet of the hen **(Colin et al., 2004)**, and the main source of pigment in conventional diets is yellow corn **(Lokaewmanee et al., 2010)**.

The main challenge of our research was evaluating the effect of supplementation of the diet of laying hens with solid-state fermented feed on egg qualitative variables. The diet of laying hens was supplemented with 10% and 15% of solid-state fermented feed by the filamentous fungi *Mortierella alpina* CCF 2861.

Scientific hypothesis

1. We assume that supplementation of solid-state fermented feed in the diet of laying hens affects the physical variables of the egg.
2. We assume that egg yolk colour of eggs will be affected by the application of solid-state fermented feed in the diet of laying hens belonging to the experimental groups.
3. We assume that solid-state fermented feed in the diet of laying hens influences antioxidant activity and extent of the lipid oxidation in egg yolk samples.
4. We assume that a higher impact will be made on qualitative variables of produced eggs with supplementation of 15% of solid-state fermented feed in the diet of laying hens, in comparison to 10% supplementation.

MATERIAL AND METHODOLOGY

The animal protocol for this research was approved by the Ethical Committee for Animal Care and Use of University of Veterinary Medicine and Pharmacy in Kosice (The Slovak Republic). The experiment was carried out in accordance with the 'European Directive on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' **(European Parliament and Council, 2010)**.

Preparation of fermented feed

Fermented feed (FF) was prepared by fungal solid-state fermentation (SSF) according to the modified method of **Čertík et al. (2006)**. The low filamentous fungal strain *Mortierella alpina* CCF 2861 was used. *M. alpina* CCF

2861 was obtained from the Culture Collection of Fungi, Charles University, Prague, Czech Republic. For the preparation of the spore suspension, which was inoculated with the SSF substrate, *M. alpina* CCF 2861 was grown for 10 days on rice. After 10 days, the spores were washed with distilled water with 0.05% Tween 80 and filtered through a gauze to remove the solid substrate. The spore suspension thus prepared was diluted to a concentration of 2.10^5 spores.mL^{-1}. Wheat bran was used as a substrate.

Birds, Housing, Diets and Experimental Design

The experiment was carried out at the University of Veterinary Medicine and Pharmacy in Košice.

For the trial, 30 laying hens Lohmann Brown classic layers aged 17 weeks, were individually weighed and divided into three groups (10 hens per pen).

The control group of laying hens (C) was fed with a commercial feed mixture without the supplementation of FF (De Heus, Bučovice, Czech Republic). Components of commercial feed mixture were corn, wheat, calcium carbonate, sunflower pomace, soya meal, rapeseed meal, wheat bran, corn gluten feed, barley, dark distillery grape, vinasse, vegetable oil and sunflower fat, monocalciumphosphate and sodium chloride. The nutrition composition of basal and experimental feed mixtures is presented in Table 1.

The first experimental group of laying hens (FF10) was fed with commercial feed mixture supplemented with 10% of FF, and second experimental group with 15% supplementation (FF15). First two weeks of acclimatization for animals were assigned to the experimental groups. Fermented feed was supplemented to the laying hens after the beginning of egg performance, in the 21st week.

Determination of egg qualitative variables

To determine egg quality, 150 eggs, collected from week 25, were used for analyses.

Egg Analyzer™ (Orka Food Technology Ltd., Ramat HaSharon, Israel) was used to determine the egg weight, Haugh units (HU), quality grade and yolk colour. The device has measured egg weight (g), the height of the thick albumen (mm), and colour of the yolk. The first two measurements were used for calculation of HU, which indicates egg quality. The equation for working out the rating is shown below: $HU = 100 \log (h - 1.7w^{0.37} + 7.6)$, where: HU = egg quality in Haugh units; w = egg weight in grams; h = height of the thick albumen in mm **(Nagy et al., 2011)**.

The air cell depth of eggs was expressed in millimetres. The percentage of shell, yolk and albumen was calculated.

Eggshell breaking force was measured in accordance with the manufacturer's instructions by the Egg force reader (Orka Food Technology Ltd., Ramat HaSharon, Israel) - a compact system for automatic measuring of the eggshell breaking point. The unit of strength measurement involves gentle application of force on the eggshell until it cracks. The results were interpreted as kilogram-force (kgf).

The pH of egg yolk and albumin were measured by the WTW 7110 pH meter (WTW GmbH, Weilheim, Germany). Antioxidant activity of yolk was detected spectrophotometrically by the method of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging **(Brand-Williams et al., 1995)**. The extent of lipid oxidation in egg yolk samples was evaluated via measurement of thiobarbituric acid reactive substances (TBARS) according to the method **Reitznerová et al**. **(2017)**. Thiobarbituric acid reactive substances (TBARS) values were measured spectrophotometrically at 532 nm (Helios c; Thermo spectronic, Cambridge, UK). The results were quantified as MDA equivalents (mg.kg^{-1}).

Colour of egg yolk was determined by Minolta Chroma Meter CR-410 (Ø 50 mm, average daily light with a colour temperature of about 6500 K (D65) (Konica Minolta, Sensing, Inc. Japan), using a programme of a Colour Data Software CM-S100w SpectraMagic™ NX (Konica Minolta Sensing Inc., Osaka, Japan, 2014). The equipment was calibrated against a standard light white reference tile and the measurements were conducted under a 2° standard observer angle. The colour parameters L* − lightness was

Table 1 The nutritional composition of laying hens diet

	FF	C	FF10	FF15
Dry matter [g.kg^{-1}]	1000.00	1000.00	1000.00	1000.00
Crude protein [g.kg^{-1}]	193.38	157.53	167.12	165.64
Crude fat [g.kg^{-1}]	50.14	37.23	41.70	38.72
Crude fiber [g.kg^{-1}]	169.07	58.88	76.15	78.42
Ash [g.kg^{-1}]	75.75	154.33	147.37	112.19
Starch [g.kg^{-1}]	75.53	411.84	353.01	371.76
Ca [g.kg^{-1}]	1.63	20.00	19.75	15.40
P [g.kg^{-1}]	5.97	4.86	5.93	4.40
Mg [g.kg^{-1}]	4.34	3.09	3.29	3.41
Ma [g.kg^{-1}]	0.22	2.21	2.41	0.99
K [g.kg^{-1}]	13.02	7.51	8.12	7.92
Cu [mg.kg^{-1}]	36.03	44.19	73.52	41.79
Zn [mg.kg^{-1}]	106.78	101.63	96.57	64.89
Mn [mg.kg^{-1}]	195.44	173.44	176.67	166.08

Note: FF – fermented feed; C – control group of laying hens; FF10 – laying hens fed with diet supplemented with 10% of fermented feed; FF15 - laying hens fed with diet supplemented with 15% of fermented feed; *SD* – standard deviation.

measured on a scale of 0 to 100%; a* − redness; b* − yellowness. Colour measurements were determined according to the CIELab colour space system **(Commission Internationale de l'Eclairage, 1986)**.

The instrument was calibrated with a white reference plate (Konica Minolta, Sensing, Inc. Japan), with setting values (L* = 97.10, a* = -4.88, b* = 7.04) before the measurement.

CIE total colour difference (ΔE*), as the distance between the two points, was calculated according to the following formula: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b)^2]^{1/2}$. Chroma C* represents a value which measures the extent to which the particular colourity differs from grey. Chroma C* was calculated according to this formula: $C^* = (a^{*2} + b^{*2})^{1/2}$. Hue h_{ab} is expressed as the name of colour and this value was calculated according to the following formula: $h_{ab} = tg^{-1} (b^*/a^*)$.

Statistical analysis

Data analysis was carried out with GraphPad Prism 8.3.0.538 (GraphPad Software, San Diego, California, USA). The effect of the supplementation of laying hens diet with solid-state fermented feed with 10% and 15% on egg qualitative variables was set as the main factor. A one-way analysis of variance (ANOVA), followed by Tukey's post-test, was conducted and a confidence interval was set at 95%.

RESULTS AND DISCUSSION

Table 2 shows the results of the physical variables of eggs produced in the 25th week. The maximum egg weight was detected in the experimental group of laying hens (53.13 ±3.74 g) after 10% addition of fermented feed (FF10). Egg yolk weight increased in the experimental group FF15 to the value of 12.24 ±1.25 g per egg. Eggshell mean weight ranged from 6.91 ±0.58 g (FF15 group) to 7.48 ±1.92 g in the eggs originating from the control group (C) of laying hens. However, no statistically significant effect on physical variables of produced eggs was observed when the experimental diet was provided (p >0.05).

The effect of solid-state fermented feed on quality of the produced eggs was examined on the fifth week of egg performance (in 25th week). The results of the qualitative variables determinations are shown in Table 3. The eggs from F10 and FF15 experimental group had a significantly higher eggshell hardness, 5.80 ±0.96 kgf and 5.69 ±0.65 kgf, respectively, than eggs produced by the C laying hens group (2.78 ±1.16 kgf), which were fed only with basic feed mixture (p <0.05). The supplementation of 15% fermented feed in diet of the FF15 group showed an increase in HU values of egg performance. The experimental supplementation in the diet of laying hens had no significant effects on egg quality traits (Table 3) in terms of yolk and albumen index and in the size of air bubbles

(p >0.05). The pH of yolk and albumen did not show significant differences between both experimental groups of laying hens (p >0.05).

The eggshell hardness was statistically different among the experimental groups (p <0.05). Maximum eggshell hardness was determined in egg samples of FF10 group, which was 2.86 times higher than in the eggs of C group (Table 3). The supplementation of laying hens with an experimental diet showed a significant effect on eggshell hardness (p <0.05).

The results of DPPH radical scavenging activity (antioxidant activity) and malondialdehyde content in egg yolk samples of produced eggs are presented in Table 4. The antioxidant activity of the egg yolk was affected by the feeding of both 10% and 15% supplementation with fermented feed. The ability to scavenge free DPPH radicals was significantly higher in egg yolk of experimental groups (FF10 and FF15) in comparison to the values obtained for the eggs from the control group (p <0.05).

Malondialdehyde (MDA) values in samples of the eggs were lower for groups FF10 and FF15 than in the control group (C), although the differences between values for this parameter did not show significant differences (p >0.05).

Colourimetric parameters of egg yolks, obtained from eggs belonging to the experimental groups, were measured by Minolta Chroma Meter CR-410 (Ø 50 mm, average daily light with colour temperature of about 6500 K (D65), standard measurement angle 2°) and are listed in Table 5.

L* variable, which represents light shade intensity (0 – black, 100 – white), exhibited a significant difference (p <0.01). A decrease in the mean value of L* variable in the FF15 indicates darkening of the egg yolk. This suggests that the egg yolk from the eggs of laying hens fed with supplementation of 15% fermented feed contains higher concentrations of colour pigment. Variable a* reflects the changes in the area of red-colour wavelengths and can reach a maximum in sharp red colour (+120) or a minimum in green-blue colour (-80). Egg yolk obtained from all experimental groups of laying hens showed no significant changes in indicator a* (p >0.05).

According to the colour measurements, descriptive egg parameters were numerically affected only by the mean of L* variable and statistically differed (p <0.01). The highest L* value was obtained with higher egg yolk colour in FF15 of experimental eggs. The presented egg yolk colour was not significantly different in a*, b*, h*, C* and index of egg yolk values, respectively.

Table 2 Physical variables of produced eggs in the 25th week of egg performance (mean ±*SD*).

	C	FF10	FF15
Egg weight [g]	52.31 ±2.71	53.14 ±3.74	51.90 ±2.57
Yolk [g]	12.50 ±0.14	12.15 ±0.94	12.24 ±1.25
Albumin [g]	32.33 ±1.86	33.62 ±3.77	32.75 ±1.04
Eggshell [g]	7.48 ±1.92	7.37 ±0.91	6.91 ±0.58

Note: C – control group of laying hens; FF10 – laying hens fed with diet supplemented with 10% of fermented feed; FF15 – laying hens fed with diet supplemented with 15% of fermented feed; *SD* – standard deviation.

The specific lightness of egg colour, e.g. indicator L*, increased significantly in FF15 experimental group (p <0.01). Variable a* (red colour) in experimental eggs remained unaffected, egg yolk showed a trend towards orange colour (Table 5). Value of variable b* slightly increased after supplementation with 10% of fermented feed in hens diet and slightly decreased with 15%. However, the corresponding differences were not significant (p >0.05).

The indicators of colourity are shown in Table 5. Hue h* slightly increased (FF10) and decreased (FF15) with fermented feed. However, the change in h* variable was not significant (p >0.05). Also, chroma C* variable (the value by which particular colourity differs from grey) does not represent significant differences among groups (p >0.05).

Table egg colour can be controlled by a subjective method, with DSM YolkFan^{TM} or an objective method, the chromameter Minolta **(Hamelin and Hernandez, 2011)**. CIE total colour difference (ΔE*) was the only indicator that differs from the control. The difference was higher for the higher dose (2.61) of fermented feed in the diet of laying hens than for the lower dose (1.96) of fermented feed, 15% and 10%, respectively. The absolute value of the difference between FF10 and FF15 groups of experimental laying hens was low, 0.65. A ΔE* value higher than 1.00 expresses that the colour difference of two samples, which are measured, is detectable with human eye.

Egg producers, consumers and processors´ perspectives have different meanings for the definition of egg quality. Easy eggshell removal and separation of the yolk from the albumen, as well as functional properties of eggs, are very important for the processors of eggs **(Alleoni and Antunes, 2001).** For the egg producers, egg quality usually means the egg weight and quality of eggshell, whereas consumers are interested mostly in shelf life, the external appearance of eggs and sensorial qualities, such as eggshell and yolk colour **(Faitarone et al., 2016; Ketta and Tůmová, 2016)**.

Several studies have been published in recent years, in which filamentous fungi in SSF feed were successfully applied in poultry nutrition. However, most published scientific articles with an application of SSF feed in poultry production are related to broilers nutrition **(Bača et al., 2014; Marcinčák et al., 2018; Sugiharto and Ranjitkar, 2019)**. On the other hand, supplementation of laying hens diet with microbial probiotics, plant additives,

Table 3 The results of qualitative variables of produced eggs (mean ±*SD*).

Variable	C	FF10	FF15
Eggshell hardness [kgf]	2.78 ±1.16^{a}	5.80 ±0.96^{b}	5.69 ±0.65^{b}
Haugh unit	65.66 ±23.19	64.42 ±31.34	71.52 ±13.90
Air cell [mm]	1.56 ±0.60	1.74 ±0.25	1.74 ±0.25
Yolk index [%]	40.71 ±6.59	39.78 ±6.38	37.32 ±3.52
Albumen index [%]	6.84 ±4.05	7.96 ±2.29	7.08 ±3.22
Yolk pH	6.03 ±0.02	6.12 ±0.03	6.06 ±0.01
Albumen pH	8.26 ±0.03	8.39 ±0.02	8.38 ±0.02

Note: C - control group of laying hens; FF10 - laying hens fed with diet supplemented with 10% of fermented feed; FF15 - laying hens fed with diet supplemented with 15% of fermented feed; *SD* - standard deviation. $^{a–b}$ - in a row means without a common superscript letter differ (p <0.05).

Table 4 The results of DPPH radical scavenging activity (antioxidant activity) and malondialdehyde content in egg yolk samples (mean ±*SD*).

Variable	C	FF10	FF15
Antioxidant activity [%]	21.68 ±1.26^{b}	24.12 ±0.30^{a}	25.18 ±0.46^{a}
MDA [mg.kg^{-1}]	0.95 ±0.13	0.89 ±0.33	0.94 ±0.13

Note: C - control group of laying hens; FF10 - laying hens fed with diet supplemented with 10% of fermented feed; FF15 - laying hens fed with diet supplemented with 15% of fermented feed; MDA – malondialdehyde; *SD* - standard deviation. $^{a–b}$ - in a row means without a common superscript letter differ (p <0.05).

Table 5 The results of colourimetric variables of egg yolk samples (mean ±*SD*).

Variable	C	FF10	FF15
L*	95.82 ±2.06^{a}	96.90 ±3.36^{a}	93.50 ±1.62^{b}
a*	33.20 ±6.77^{a}	32.03 ±5.47^{a}	34.340 ±3.37^{a}
b*	115.55 ±6.11^{a}	116.69 ±8.61^{a}	115.89 ±2.69^{a}
C*	120.35 ±7.33^{a}	121.22 ±7.13^{a}	120.92 ±2.82^{a}
h*	74.09 ±2.64^{a}	74.47 ±3.47^{a}	73.50 ±1.54^{a}
YI	142.72 ±7.57^{a}	141.47 ±3.83^{a}	145.21 ±3.94^{a}

Note: C – control group of laying hens; FF10 – laying hens fed with diet supplemented with 10% of fermented feed; FF15 – laying hens fed with diet supplemented with 15% of fermented feed; L* – lightness; a* – red/greenness; b* – blue/yellowness; C* – chromaticity; YI - index of yellow colour according to the standard DIN 6167; *SD* – standard deviation. $^{a–b}$ – in a row means without a common superscript letter differ (p <0.05).

etc. were also studied. The influence of probiotic preparation based on lactobacillus, oregano essential oil, sumac (*Rhus coriaria*), propolis and pollen on egg quality parameters of Lohmann hybrid laying hens were studied by the authors **Arpášová et al. (2012)**, who observed that supplementation did not negatively affect monitored egg quality parameters. Influence of dietary inclusion of *Bacillus licheniformis* on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens was also studied **(Lei et al., 2013)**. The quality of the table eggs, their damage and spoiling in various age of the laying hens during the second phase of the laying cycle was studied by the authors **Angelovičová, Ševčíková and Angelovič (2015)**, who assumed that the values of egg shell weight were not directly related to egg weight and egg white weight.

Nevertheless, egg quality remains an interesting subject to investigate. Organoleptic properties and consumption of eggs are associated with the egg quality variables. **Kozelová et al. (2018)** examined the opinions of the Slovak consumers about the purchase and consumption of eggs and identified their preferences at egg purchase.

A wide variety of scientific literature investigates how the quality and composition of eggs can be altered concerning their use for human nutrition. These changes are often caused by the diet, using some specific ingredients in the feed of laying hens to reach a change in the profile of yolk lipids, mostly to improve the content of lipid fatty acid composition **(Koreleski et al., 2003; Faitarone et al., 2016)**. On the other hand, if the diet of laying hens contains sources of PUFA, e.g. via vegetable oil supplementation, the yolk of eggs can present high lipid oxidation, when compared with those derived from laying hens fed a diet without supplementation. **Faitarone et al. (2016)** stated in their work that the analysis of variance showed significant differences in yolk lipid oxidation values in eggs laid by white layers fed diets supplemented with different vegetable oils and their concentrations (e.g. linseed, canola, soybean oils and their different mixtures). In this experiment, it was detected, that after 10 days of storage at room temperature, the eggs laid by hens fed a diet with no oil supplementation or supplemented with 2.5% soybean oil showed a lower degree of yolk lipid oxidation in comparison to the eggs laid by hens fed diets supplemented with 5% linseed oil and with 2.5% canola oil +2.5% soybean oil. These results were not significantly different from the other treatments. **Giampietro et al. (2008)** stated that yolk lipid oxidation increased according to egg age, for instance: 0.1343 TBA values in fresh eggs *versus* 0.1698 in eggs stored for seven days, and 0.2138 in eggs stored for 14 days, respectively. According to **Koreleski et al. (2003)**, TBARS values in eggs stored for 15 days at a temperature from 4 to 8 °C were significantly higher for control group I (basal diet) and II than in the other groups. The basal diet in those groups was supplemented with 0.3% Lyso fish fat (groups II, III, IV and V), while reducing the proportion of blended fat by the same amount. So, the lowest yolk fat oxidation was detected in eggs of hens fed the diet supplemented with synthetic antioxidant or vitamin E, and then this was followed by the group supplemented with vitamin C.

Yolk colour was determined as the mean of five readings in the centre of the yolk of each egg by colourimeter Minolta CR-400. The colour of egg yolk is often related to the egg quality and it is an important biophysical parameter. Yolk colour depends on the chemical and physical properties of the light source (wavelength and intensity) and the actual ability of the observer to perceive colour. Determination of yolk colour in the CIELab system is therefore deemed to be much more beneficial, because information on both colour hue and lightness can be obtained, and such parameters give a good idea of colour changes **(Dvořák et al., 2007)**.

Many scientific works deal with investigating the effect of housing and feeding on the quality of eggs as well as on the yolk colour. **Dvořák et al. (2009)** refers that, during a 7-month period of monitoring, value b* for egg yolk colour increased significantly from the 3rd month under the deep litter system of rearing. **Kopřiva et al. (2014)** reported that the addition of dried beetroot at the amount of 1 and 2% per feeding dose caused a significant increase only in specific lightness (value L*). However, lighter egg yolks did not show significant changes in CIELab colour space for values a* and b*. This is in agreement with our study.

Therefore, different intensities of yellow are induced by feeding with different feed ingredients. For example, hens fed mash diets containing yellow maize and alfalfa meal laid eggs with dark yellow yolks, while diets based on cereals such as wheat, barley or rice need dietary maize to obtain deep colour **(Lokaewmanee et al., 2009)**. The effect of different levels of marigold and paprika on egg production and yolk colour was also observed by the authors **Spasewski et al. (2017)**. Among yolk sensorial attributes, its colour is considered as a quality indicator, and it, therefore, plays an important role in egg acceptance by the consumers. Higher yolk colour intensity increases egg acceptance by the consumers, who associated more intense yolk pigmentation with higher nutritional value **(Silva et al., 2000; Schreiner et al., 2004)**.

It was concluded that the inclusion of vegetable oils in commercial white layer diets does not significantly change egg yolk pigmentation, as colourimetrically evaluated. The yolks of the eggs laid by layers fed diets containing sources of PUFA presented high lipid oxidation, particularly when compared with those derived from layers fed a diet with no oil supplementation.

Lipid stability is also vital to evaluate, as the yolk fatty acids may suffer lipid oxidation during storage. Lipid stability, as another important egg quality parameter, affects food quality, particularly in aroma, taste and nutritional value, as well as in the production of toxic compounds **(Faitarone et al., 2016)**. Fatty acids, particularly unsaturated fatty acids, are the compounds most susceptible to oxidation **(Fennema, 2000)**. **Cherian et al. (2007)** reported that the inclusion of PUFA in layer diets may increase the susceptibility of eggs to lipid oxidation.

CONCLUSION

In this experiment, the effect of supplementing the diet of laying hens with solid-state fermented feed produced by the low filamentous fungal strain *Mortierella alpina* CCF 2861 on egg qualitative variables was observed. Based on the obtained results, we can conclude that supplementation of laying hens diet with SSF feed (10% and 15%)

positively influenced the quality of eggs in the 25th week of age of laying hens. Further studies are nonetheless necessary to investigate the effect of fermented feed on microbial, physical, and chemical properties of eggs, including the fatty acids profile of eggs. Multiple factorial analysis is a suitable method for further investigation.

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THE EFFECTS OF NUTRITIONAL SUPPORT ON SELECTED LABORATORY PARAMETERS IN PATIENTS WITH COLORECTAL CANCER UNDERGOING SURGICAL RESECTION OF THE COLON

Mária Servátková, Peter Chlebo, Zuzana Chlebová

ABSTRACT

The benefit of the nutritional support provided to patients with colorectal cancer who have undergone the planned resection of the colon in relation to the laboratory markers of nutrition was examined. And it is currently being discussed, that pre-operative optimization of nutritional status reduces the incidence of post-operative complications in cancer patients and regulates selected laboratory parameters. This was a retrospective study where the treatment group (n = 52) received the enteral nutritional support 21 days before the scheduled surgery and the other group was formed of patients without preoperative enteral nutrition (n = 52). Laboratory parameters (CRP, leukocytes, albumin, total proteins) were monitored for at least one month before the planned surgery and just before the operation, and the effect of supplemental enteral nutrition on selected laboratory parameters between these two groups was compared. In a group of patients with enteral nutrition, serum albumin levels increased significantly, while CRP was significantly reduced during preoperative enteral nutrition (albumin S-ALB from 35.42 to 37.48, p = 0.0008, C reactive protein from 26.5 to 14.092, p = 0.0007). Nutritional support 21 days before surgery in oncological patients resulted in an improvement in laboratory parameters compared to the group of patients without nutritional enteric support. Malnutrition in patients who are candidates for major surgical intervention is a risk factor for postoperative morbidity and mortality. However, further studies are required to verify the effectiveness of this early nutritional intervention on medium and long-term clinical parameters in different types of cancer.

Keywords: nutridrink; enteral nutrition; malnutrition; colon; hypoalbuminemia

INTRODUCTION

Cancer of the colon and rectum is one of the most commonly diagnosed cancers in the world **(Torre et al., 2015)**. Although patients with early colorectal cancer could be successfully treated with surgical procedures, the large surgery itself may cause the dysfunction of homeostasis, defensive mechanisms, and inflammatory response, which may increase the rate of postoperative complications and extended hospital stay. Nutritional status is a key factor affecting clinical outcomes in patients **(Xu et al., 2018)**. Unintentional weight loss in cancer patients is an alarming constitutional change predicting the progress of the illness and shortened survival time **(Chow et al., 2020)**. It is important to provide nutritional support administered through enteral nutrition or parenteral nutrition **(Chow et al., 2020)**. Historically these approaches are accompanied by concerns with increased complications and costs. Therefore, enteral nutrition can be the preferred form due to its lower costs, less complications, and improved results **(Altintas et al., 2011)**.

Scientific hypothesis

We examine in our study, whether the administration of Nutridrink compact rises the level of albumin and lowers the level of C-reactive protein in patients with colon cancer before the planned surgery.

MATERIAL AND METHODOLOGY

Study design

The study was a retrospective one focused on individual cases that used the database of the surgical department of the Topoľčany city hospital in Slovakia. Data were collected from medical documentation of surgical department patients, including basic characteristics, laboratory, and perioperative data. This study was approved by the Ethics Commission of the Institute – World of Health, hospital Topoľčany. In Topoľčany hospital, it is recommended that patients with colorectal cancer should take enteric nutrition as part of preoperative optimisation of the nutritional condition before the planned colon resection.

Patient selection

The study ran from September 2015 to September 2019 and the criteria for patient spooling were as follows: age between 18 – 90 years, the diagnosis of colorectal cancer, the patient was subjected to a colorectal surgical procedure. All patients signed informed consent. The exclusive criteria were as follows: inadequate data on the patient for analysis, disapproval of the patient, non-compliance with the treatment regimen, patients who have undergone much greater surgical performance than originally planned, and patients who had already had a stoma and have previously undergone surgery for colorectal cancer, patients who have had a renal and hepatic failure, inability to consume food orally, psychiatric disorders, pregnancy, uncontrolled infection, and all patients who did not have the criteria for inclusion.

Grouping

In our database, 142 patients were identified, of which 38 patients were excluded because they did not meet the criteria for inclusion and had incomplete medical documentation and the remaining patients were divided into two groups. The treatment group was formed by 52 probants who accepted the proposal of the treating surgeon and received commercially available nutrition (Nutridrink Compact) at least 21 days before the planned surgical procedure as an addition to a normal rational diet. The patient's cooperation is an important factor in the treatment of enteral nutrition. Many patients poorly tolerate enteral nutrition and refuse to leave their normal diet. This resulted in a control group of patients who formed 52 probants who had rational nourishment as usual, and were verbally guided for nutrition.

Preoperative nutrition

Patients received Nutridrink Compact (Nutricia) as an addition to a normal rational diet, it is a high-energy nutritionally complete liquid designed for a dietetic procedure for malnutrition that is related to the disease. The content of Nutridrink is mentioned in Table 1.

Clinical evaluation

The following data were collected: body weight, height, a general state of health, age, gender, family status, degree of education, the onset of disease, number of symptoms, metastasis, co-morbidity.

Laboratory evaluation

The course of sampling is an important prerequisite for an objective assessment of the patient´s health. The sampling from the patient was realized once by a qualified nurse. Venous sampling was done in the morning after 8 – 12 hours long fasting. The patient was allowed to drink only a small amount of clear water. The blood sample was taken from the peripheral vein of the upper limb. The skin was disinfected with eighty percent ethanol. The blood was taken into the vacuum test tube, the test tube was filled to the mark and subsequently, the sample was mixed and labeled with the personal data of the patient. The material was transported into the laboratory of the company Medirex. The company Medirex is a holder of a certificate of conducting STN EN ISO 9001:2009 and simultaneously of the needed sampling certifications for individual examinations, namely hematological and biochemical. Subsequently, the result of the examination of the patient was recorded into the patient´s medical record.

Blood count, C-reactive protein, albumin, total proteins, and glycemia were recorded.

Assessment of the status of nutrition

Blood was collected minimally one month before the planned operation in both groups. In the treatment group, a high-energy nutritional supplement was added to the patient's rational diet for at least 21 days. Subsequently, blood was collected again just before surgery. The patient's weight was determined at least one month before surgery and the second time just before surgery in both patient groups.

Surgical procedures

The surgery was performed by one surgeon who specializes in the given disease of the colon by laparotomic and laparoscopic surgical techniques. The anastomosis was end-to-end or side-to-side depending on the location and the decision of the surgeon.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010. Quantitative data are expressed as mean ± standard deviation and compared with the t-test. p-value $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

A total of 142 patients were analysed, 38 were excluded because they did not meet the desired criteria for patient selection for our study, and subsequently 104 patients underwent evaluation.

The main characteristics are listed in Table 2. The female gender was 48.08% (n = 25) and male 51.92% (n = 27) in the treatment group. In the control group the female gender was 44.23% (n = 23) and male 55.77% (n = 29). The average age in the first group was 57.46 ±7.92 years and in the control group 57.92 ±8.27 years. Patients were most commonly married, 57.69% (n = 30) and 53.85% (n = 28) in the control group. The majority of patients in the treatment group completed secondary education, 73.07% (n = 38) and 80.76% (n = 42) in the control group.

The clinical characteristics of patients are mentioned in Table 3. The onset of the disease was noted just before surgery, where the first group of patients had symptoms at 57.69% (n = 30) 4 – 6 months ago, at 23.07% (n = 20) 7 – 12 months ago, at 9.61% (n = 5) 0 – 3 months ago, for 9.61% (n = 5) over 13 months ago. In the control group, 21.15% (n = 11) 0 – 3 months ago, 11.53% (n = 6) 4 – 6 months ago, 53.85% (n = 28) 7 – 12 months ago and 13.46% (n = 7) over 13 months ago. The most common stage of colorectal carcinoma reported prior to surgery was the stage II in the treatment group, 48.07% (n = 25), Stage I 19.23% (n = 10), Stage IV 19.23% (n = 10) and Stage III 13.46% (n = 7). In the control group the stage I 13.46% (n = 7), Stage II 19.23% (n = 10), Stage III 48.07% (n = 25) and stage IV 19.23% (n = 10).

Table 1 Nutritional composition of Nutridrink compact.

Energy value	1010 kJ	Minerals and trace elements	
kcal	240	Na	96 mg
Fats (35 En%)	9.3 g	K	236 mg
of this		Cl	91 mg
Saturated fatty acids	0.9 g	Ca	174 mg
Carbohydrates (49 En%)	29.7 g	P	174 mg
of this		Mg	33 mg
Sugars	15.0 g	Fe	3.8 mg
Lactosis	<0.5 g	Zn	2.9 mg
Fiber (0 En%)	0 g	Cu	0.43 mg
Proteins (16 En%)	9.6 g	Mn	0.80 mg
Salt	0.24 g	F	0.20 mg
Vitamins		Mo	24 μg
Vit A	240 μg RE	Se	14 μg
Vit D_3	1.8 μg	Cr	16 μg
Vit E	3.0 mg-α-TE	I	32 μg
Vit K	13 μg	Other	
Thiamine	0.40 mg	Choline	88 mg
Riboflavin	0.40 mg	Osmolarity	790 $mOsmol.L^{-1}$
Niacin (4.3 mg-NE)	2.2 mg		
Pantothenic acid (B_5)	1.3 mg		
Vit B_6	0.40 mg		
Folic acid	64 μg		
Vit B_{12}	0.70 μg		
Biotin	9.6 μg		
Vit C	24 mg		

Table 2 Social and demographic characteristics of patients before surgery.

		Group 1	Group 2
		N = 52	N = 52
Gender	male	27 (51.92%)	29 (55.77%)
	female	25 (48.08%)	23 (44.23%)
Aggregated age	average	57.46 SD ±7.92, median 57	57.92 SD ±8.27, median 58
Marital status	married	30 (57.69%)	28 (53.85%)
	divorced	5 (9.61%)	7 (13.46%)
	widower / widow	12 (23.07%)	11 (21.15%)
	single	5 (9.61%)	6 (11.53%)
Degree of education	analphabet	0	0
	High school	38 (73.07%)	42 (80.76%)
	University	11 (21.15%)	10 (19.23%)
	postgraduate	3 (5.76%)	0

Metastases occurred in the first group in 20 patients, representing 38.46% and in the control group in 7 patients (13.46%). 73.07% of patients had the mild systemic disease before surgery, 13.46% had a severe one and 13.46% were without systemic disease in the first group. In the control group, before surgery, 80.76% had mild systemic disease, representing 42 patients, three patients had severe systemic disease, representing 5.76%, and 7 patients had no systemic disease, representing 13.46%. The average height of the whole group of patients was 1.60 ±9.09 meters, weight 71.6 ±11.64 kilograms a month before surgery, and 71 ±12.61 kilograms just before surgery. The average weight in the first group of patients one month before surgery was 72 ±7.45 kilograms, just before surgery 73 ±12.85 kilograms and in the second group 71.9 ±10.87 kilograms a month before surgery and 71.5 ±9.64 kilograms just before surgery.

Laboratory variables

Laboratory variables are shown in Table 4. Changes in variables were reported, that means laboratory parameters of haemoglobin, leukocytes, albumin, total proteins, CRP, glycaemia in patients in initial state with enteral nutrition and just prior to surgical procedures, and then compared with the control group.

Table 3 Clinical characteristics of patient before surgery.

		Treatment group	Control group
Onset of illness in months	0 – 3	5 (9.61%)	11 (21.15%)
	4 – 6	30 (57.69%)	6 (11.53%)
	7 – 12	12 (23.07%)	28 (53.85)
	>13	5 (9.61%)	7 (13.46%)
stage	I.	10 (19.23%)	7 (13.46%)
	II.	25 (48.07%)	10 (19.23%)
	III.	7 (13.46%)	25 (48.07%)
	IV.	10 (19.23%)	10 (19.23%)
metastasis	no	32 (61.53%)	45 (86.53%)
	yes	20 (38.46%)	7 (13.46%)
comorbidity	mild systemic disease	38 (73.07%)	42 (80.76%)
	severe systemic disease	7 (13.46%)	3 (5.76%)
	without systemic disease	7 (13.46%)	7 (13.46%)

Table 4 Laboratory variables.

		Treatment group		
	Reference value	**Initial status**	**Status just before surgery**	***p*-value**
Haemoglobin Hb ($g.L^{-1}$)	Male (130 – 197) /Female (120 – 160)	114.2 ±18.04	116.90 ±16.26	0.0168
Leukocytes WBC ($10^9.L^{-1}$)	3.8.2010	8.72 ±3.6	7.74 ±3.168	0.0025
Albumin S-ALB ($g.L^{-1}$)	32 – 48	35.42 ±6.92	37.48 ±6.38	0.0008
Total proteins S-CB ($g.L^{-1}$)	57 – 82	56.02 ±6.43	61.1 ±8.7	0.0002
CRP-C reactive protein ($mg.L^{-1}$)	0 – 5	26.5 ±35.00	14.092 ±15.38	0.0007
Glycemia S-Glu ($mmol.L^{-1}$)	4.0 – 5.5	7.3 ±2.93	6.87 ±2.21	0.0153

Table 5 Postoperative complications.

	Treatment group	Control group	*p*-value
Septic complications	4%	25%	0.04
Total complications	21%	29%	0.51

In patients receiving enteral nutrition, the albumin and total proteins increased, which was statistically significant and there was a decrease in the number of leukocytes and CRP, statistically significant, and also glycemia was adjusted. In the treatment group, the haemoglobin changed from 114.2 to 116.90, $p = 0.0168$, leukocytes WBC from 8.72 to 7.74, $p = 0.0025$, albumin S-ALB from 35.42 to 37.48, $p = 0.0008$, total proteins S-CB from 56.02 to 61.1, $p = 0.0002$, CRP-C reactive protein from 26.5 to 14.092, $p = 0.0007$, glycemia S-Glu from 7.3 to 6.87, $p = 0.0153$. In the control group, the haemoglobin changed from 113.0 to 115.61, $p = 0.0097$, leukocytes WBC from 8.25 to 7.98, $p = 0.0973$, albumin S-ALB from 33.15 to 33.57, $p = 0.1073$, total proteins S-CB from 57.1 to 58.94, $p = 0.00108$, CRP-C reactive protein from 10.6 to 10.45, $p = 0.247$, glycaemia S-Glu from 6.31 to 5.85, $p = 0.027$. In the control group, there was a change in total protein, which was statistically significant.

Postoperative complications

Postoperative complications within 30 days are listed in Table 5. The number of septic complications was significantly lower in patients in the treatment group with enteral nutrition than in patients in the control group (4% compared to 25%, $p = 0.04$). There wasn´t a significant difference in the number of total complications between the two groups (21% compared to 29%, $p = 0.51$).

This study retrospectively examined the association of laboratory parameters, namely albumin, total proteins, leukocytes, CRP, glycemia, and preoperative enteral nutrition in patients who underwent a resection of the colon for malignancies. In our study, we reported adding Nutridrink to patients with the oncological disease for at least 21 days, which increased serum albumin, total proteins, and reduced CRP levels in patients undergoing planned colon resection. This preoperative preparation can be a useful strategy as a preoperative method to improve postoperative forecasts in patients. The systemic score of

inflammation and nutrition plays an important role in various cancers in certain situations. The reports have shown that inflammation promotes the invasion of tumors and metastases through the activation of IL-6 and T-lymfocytes **(Tokunaga et al., 2017)**. Our study showed that the addition of Nutridrink to treatment before surgery reduced CRP levels and increased serum albumin of total proteins in patients with colorectal cancer who had undergone a therapeutic resection of the colon. We believe that our result is remarkable because our two groups have been well aligned with respect to the basic demographic parameters, thus reducing confusing variables. The surgical variability was reduced because the surgical procedure was performed by one surgeon in both groups.

There are some limitations to this study. Firstly, our set of patients was small, second, nutrition disorders in cancer patients occur not only by cancer pathologies but also by preoperative chemotherapy or radiotherapy, thirdly, our study was retrospective and was performed on one institution. Nevertheless, it is a study, which in Slovakia is one of few comparing the effect of enteral preoperative nutrition in a patient with colorectal cancer undergoing surgical resection. Serum albumin levels are traditionally used as a biochemical indicator of individual nutritional status before surgery. It is considered to be the exact preoperative prognostic indicator for various surgical performances including cardiac, trauma, and general surgery **(Truong et al., 2016)**. While some believe that low albumin levels indicate malnutrition, others assume that hypoalbuminemia stems from a state of chronic disease, and this a resulting inflammation and is not caused by malnutrition, thereby preventing any beneficial effects of nutritional therapy **(Truong et al., 2016)**. With respect to serum albumin levels, this value gradually decreases between 0.08 and 0.17 $g.l^{-1}$ per year with age. In addition, elderly patients are usually complicated by comorbidities that increase inflammatory cytokines, and both loss of appetite and loss of muscle mass directly cause hypoalbuminemia **(Tominaga et al. 2019)**.

However, enteral and parenteral nutrition have been shown to improve the results in undernourished patients undergoing large elective surgical intervention **(Braga et al., 2002)**. The agreement is to stabilize the baseline nutritional status and administer the enteral or parenteral nutrition to the severely hypoalbuminate patients before surgery, even if it requires the delay of surgery. In 2012, **Oberhofer et al. (2012)** showed an increase in CRP in the early postoperative period after colorectal tract surgery correlated with a significant increase in complication rates (p <0.001), which was consistent with Welsch **(Welsch et al., 2007)**, who showed that CRP values greater than 140 $mg.l^{-1}$ on a postoperative day 3 or 4 predicted infectious complications and anastomotic leaks after colorectal surgery. In contrast, preoperative CRP levels did not correlate with the incidence of postoperative complications **(Truong et al., 2016)**. CRP is an acute-phase protein that is synthesized in the liver in response to proinflammatory cytokine signaling, primarily through interleukin-6 and alpha tumor necrosis factor. An important role of CRP is to bind phosphocholine to pathogens as well as to apoptotic or necrotic host cells, which in turn activate the complement system and obtain phagocytes.

Serum CRP increases rapidly in response to tissue damage or infection, but an increase in CRP (generally at low levels) is also seen in chronic inflammatory or neoplastic conditions, a process likely to be mediated by various signaling mechanisms **(Crockett et al., 2014)**. **Platt et al. (2012)** reported data on WBC, CRP, and albumin concentrations in 454 patients undergoing colorectal cancer surgery, of which 104 developed infectious complications. The results showed that CRP measurements after resection for colorectal carcinoma accurately predict infectious complications including anastomotic leakage.

The average time to onset of infectious complications, including anastomotic leakage, was 6 – 8 days after surgery. Colorectal surgery has traditionally been associated with significant morbidity and prolonged hospital stay. The overall complication rate was reported to be 26 – 35%. In particular, infectious complications are a major cause of morbidity and mortality after colorectal surgery **(Sonoda et al., 2015)**. Albumin is considered a negative protein in the acute phase because its concentration decreases during injury and sepsis. In patients with septic shock, the rate of albumin loss in tissue spaces increases by more than 300%. Hypoalbuminaemia is a risk factor for mortality and postoperative complications. Therefore, nutrition control is an important focus of perioperative management. The magnitude of the systemic inflammatory response during the perioperative period, as indicated by acute-phase proteins – in particular C-reactive protein (CRP) – can help identify the risk of postoperative infectious complications.

A correlation was reported between serum albumin and CRP with gastrointestinal cancer **(Feng, Zhao and Chen, 2014)**.

Anastomosis leakage after rectal surgery is one of the most serious and life-threatening complications and still poses a main clinical problem **(Welsch et al., 2007)**. Up to 50% of patients with anastomotic leakage are asymptomatic, which can be explained by extraperitoneal localization of leakage. Postoperative mortality on anastomotic complications is up to 22% and it is estimated, that it constitutes one-third of all deaths after colorectal surgery **(Nesbakken et al., 2005)**. New recommendations for operations and oncology were published in 2016 and 2017. Nutritional intervention before major surgery in patients with malnutrition should be made for at least 10 – 14 days, even at the cost of defering the operation (at least 7 – 14 days) and this is a strong recommendation. Enteral nutrition is the preferred way of feeding. Parenteral nutrition is recommended only if the patient cannot be fed through the digestive tract **(Weimann et al., 2017; Arends et al., 2017)**. However, enteral and parenteral nutrition has been shown to improve the results in undernourished patients undergoing large elective surgical intervention **(Braga et al., 2002)**. Undoubtedly the use of enteral nutrition reduces the number of perioperative complications and decreases the time spent in the hospital **(Heyland et al., 2001)**. It should be remembered that it is very important to continue the nutritional intervention even after the operation **(Klek et al., 2011)**. For example, Giger-Pabst **(Giger-Pabst et al., 2013)**, found that preoperative peroral supplementation

with an immune-enriched diet for 3 days did not improve postoperative outcome in patients with gastrointestinal cancer and meanwhile, no positive effects of immunonutritional support were found in patients on ICU. **(Atkinson, Sieffert and Bihari, 1998; Bower et al., 1995)**. The study, which included 1223 critically ill adults, also showed the deleterious effect of early immunonutritional administration **(Heyland et al., 2013)**. In the past, the originally popular parenteral nutrition was replaced by enteral feeding in the early 1990s. Many experts have supported changes in the nutritional approach, especially because there is a lack of evidence-based data. Few authors dared to express their doubts about the enteral technique, including difficulties in administering the required dose or the occurrence of complications **(Braga et al., 1999; Gianotti et al., 2000; Gianotti et al., 2002; Gianotti et al., 1997; Heslin et al., 1997; Klek et al., 2008)**. The management of malnutrition in patients with metastatic carcinoma belongs to the complex management of illness, so the tolerance and effectiveness of ever more aggressive treatment increases and the life quality of patients improve as well **(Gallois et al., 2019)**.

CONCLUSION

In conclusion, our study shows that albumin, total protein, CRP may be a useful marker in colorectal cancer patients suffering from malnutrition. In addition, further extensive studies are needed to evaluate the clinical utility of enteral preoperative nutrition and nutritional markers.

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THERMAL STABILITY OF PREPARED CHICKEN FEET GELATINE GEL IN COMPARISON WITH COMMERCIAL GELATINES

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ABSTRACT

Gelatine is, due to its functional properties, currently widely used not only in the food industry (in the production of confectionery, dairy products, canned food) but also in pharmacy (soft and hard capsules) and cosmetics (creams, lotions) where it applies its ability to form thermoreversible gel stronger than most other gelling agents. What is more, it provides further excellent properties including emulsifying, foaming, stabilizing, film-forming, water and fat binding, texturizing, thickening, and adhesive attributes which makes it a very important hydrocolloid. Gelatine is obtained from the raw material of animal tissues containing collagen, usually mammalian skin or bones. For religious reasons in some countries, pork or bovine gelatine must be replaced by an alternative form, such as poultry or fish gelatine. The quality of gelatine is assessed mostly by the strength of gelatine gel which strongly depends on ambient temperature or humidity. Extraction conditions may also significantly affect the quality of gelatine. This study examined possible changes in the strength of gelatine gels prepared from laboratory-produced chicken feet gelatine and compared them with commercially available pork and beef gelatines at temperatures of 23, 29, and 35 °C at 60 and 80% humidity. While at 23 °C thermal stability of prepared chicken gelatine was monitored higher than in commercial gelatines, experiments at 29 and 35 °C provided equivalent results for chicken and commercial gelatines. Therefore, prepared chicken gelatine offers a significant potential to become an alternative to traditional gelatines. The information about gelatine gels thermal stability is of great importance for applications not only in the food; but also in the pharmaceutical industry.

Keywords: chicken feet; collagen; gelatine; gel strength; poultry by-products; thermal stability

INTRODUCTION

Gelatine is a multifunctional biopolymer acknowledged as a functional food due to its positive effects on human health **(Jellouli et al., 2011)**. The application of gelatine dates back to 4000 B.C. when Egyptians used glue based on gelatine to connect parts of furniture **(Koepff, 1985)**. At the end of the seventeenth century, commercial production of gelatine commenced. More than a hundred years later, the production process significantly enhanced and high molecular weight gelatine was produced. Thus, consequently, the high quality of gelatine gels was obtained **(Bogue, 1922; Smith, 1929)**. At present, annual gelatine production comprises approximately 583.400 tonnes per year worldwide **(Grand View Research, 2020)**.

Gelatine is acquired by thermal denaturation or partial hydrolysis of materials containing collagen which is **(Mohtar et al., 2010)** a fiber-forming protein important for maintaining the structure of animal tissues **(Li et al., 2009)**. It is the most widespread protein in mammals accounting for up to 30% of all proteins **(Perez-Tamayo, 1978)**. Scientific literature states that 29 different types of collagen have been currently identified **(Silvipriya et al., 2015)**. Collagen (type I) is an insoluble fibrous structural protein abundant (about 25%) in animal tissues, such as skins, bones, tendons, ligaments, and cornea **(Maroušek et al., 2015; Krishnamoorthi et al., 2017)**. Collagen possesses significant properties including high tensile strength, low antigenicity, and good biocompatibility **(Subhan et al., 2017)**. The collagen molecule is comprised of three polypeptide chains that form a helical structure. Chains conformation is changed during gelatine gelation and a three-dimensional network structure similar to the natural arrangement of collagen is created **(Bigi et al., 2004)**. Gelatine has a lower molecular weight than native collagen because it is composed of a mixture of polypeptide segments with a molecular weight in the range of 16 – 150 kDa **(Asghar and Henrickson, 1982)**.

Physical properties of gelatine, such as gel-forming ability, water holding capacity, fat binding capacity, or emulsifying and foaming properties, are of great significance in applications particularly in the food industry within many products, such as marshmallows, jellies or gummy bears. Thermal stability of gelatine at temperatures between 25 and 30 °C is also principal in

gelatine desserts or in applications of gelatine in combination with other hydrocolloids including agar-agar gels in fruit gummies where it is essential to maintain required clarity or textural properties **(Schrieber and Gareis, 2007)**. According to the analysis by **Grand View Research (2020)**, gelatine is applied most as a stabilizer (238.000 tonnes), thickener (186.000 tonnes) and gelling agent (147.000 tonnes) which emphasizes the fact that thermal stability of gelatine gels is one of the most significant required gelatine attributes. These functional properties primarily affect the texture and appearance of the final products **(Li et al., 2009)**. Gelatine´s ability to retain molecules of water is advantageously utilized also in cosmetic formulations **(Deyl et al., 2003)**. Considering these valuable characteristics, gelatine is also extensively used in biomedicine and pharmaceutical industry **(Bae et al., 2008)**. Gelatine has antimicrobial or antioxidant properties providing the ability to act as an anti-hypertensive agent via angiotensin inhibition (ACE) **(Gómez-Guillén et al., 2011**). Several studies have revealed that gelatine and especially gelatine hydrolysates exhibit regenerative effects on the human skeleton and spinal cord systems **(Schrieber and Gareis, 2007)**.

One of the most important quality indicators is gelatine gel strength (Bloom value). Reflecting its value, gelatine is classified into low gel strength gelatine (≤150 Bloom), medium gel strength gelatine (150 – 220 Bloom), and high gel strength gelatine (220 – 300 Bloom**) (Johnston-Bank, 1983)**. Gelatine gel strength may depend on several different factors, such as the age, species, and gender of the animal used as raw material, technological conditions of gelatine preparation, including pre-treatment method, extract temperature and time, and the ambient conditions - pH, temperature and humidity.

The thermal stability of different types of gelatines has been thoroughly examined. **Losso and Ogawa (2013)** determined the thermal stability of chicken keel bone collagen. **Pati et al. (2010)** isolated and characterized fish scale collagen of higher thermal stability. In both studies, thermal stability was established using denaturation temperature. **Michon et al. (1997)** investigated the influence of thermal history on the stability of gelatine gels using the DSC method. **Rodriguez-Rodriguez et al. (2019)** studied the development of thermal stability of gelatine/chitosan/PVA hydrogels. To determine the thermal stability of gelatines, the DSC method and rheological testing were applied. **Canpanean et al. (2013)** examined the enhanced thermal stability of gelatine coated gold nanorods in water solution. Thermal stability was established using UV-visible spectrophotometry and TEM microscopy. **Masutani et al. (2014)** monitored increased thermal stability of gelatine films by UV-induced cross-linking with glucose applying the DSC method, SEM microscopy, and UV-visible spectrophotometry. Cross-linking is one of the three methods to enhance the thermal stability of gelatines. **Rodriguez-Castellanos et al. (2014)** examined nanomechanical properties and thermal stability of recycled cellulose reinforced by a starch-gelatin polymer composite using SEM microscopy and TGA analysis. However, very little data deals with the thermal stability of gelatine gel strength which is a key parameter to evaluate gelatine quality in food, such as confections, aspics, dairy, and meat products. Therefore, this study focuses on the changes in gel strength of products containing gelatine gel during the storage at different temperatures.

THE AIMS OF THIS STUDY

The study aims to continue the research of biotechnological processing of poultry by-products into gelatine **(Mrázek et al., 2019)** and to prepare chicken feet gelatine (CFG), beef, and pork gelatine gels. Furthermore, to monitor an impact of temperature and humidity on CFG gels during the storage at different temperatures of 23, 29 and 35 °C as a simulation of standard storage conditions suitable for food products containing gelatine, and simultaneously, as a simulation of storage conditions in summers in the moderate climate zone and subtropical or tropical areas. It also provides tests at an increased relative humidity (60 and 80%) since gelatine gels are commonly stored in cooling facilities with humidity often up to 80%. It compares CFG with commercially available pork and beef gelatine.

Scientific hypothesis

The research has tested the hypothesis that the thermal stability of gelatine gels decreases with rising temperature and humidity.

MATERIAL AND METHODOLOGY

Appliances, tools, and chemicals

Stevens LFRA Texture Analyser for measuring gelatine gel strength (Leonard Farnell and Co Ltd., England), P 98 meat mincer (Brather, Spain), Memmert ULP 400 drying device (Memmert GmbH + Co. KG, Germany), LT 43 shaker (Nedform, Czech Republic), Kern 440 – 47 electronic scale, Kern 770 electronic analytical scale (Kern, Germany), A 10 labortechnik analytical mill (IKA-Werke, Germany), ULP 400 drying oven (Memmert GmbH+Co. KG, Germany), SLR heating board (Schott Gerate GmbH, Germany), Whatman No. 1 paper (Sigma Aldrich, UK), a metal filter sieve with the size of pores 1 and 2 mm (Labor-komplet, Czech Republic). Chemicals: NaCl, NaOH, petroleum ether, ethanol, and chloroform (Verkon, Czech Republic); all chemicals were of analytical grade. Proteolytic enzyme Polarzyme 6.0 T – serine endoprotease manufactured by fermentation of microorganisms that are not present in the final product (Novozymes, Denmark) with the declared enzyme activity of 6 KPU per g (kilo protease unit per g). Commercial mammalian gelatines: pork gelatines with the gel strength of 212 and 288 Bloom, beef gelatines with the gel strength of 266 and 273 Bloom.

Preparation and measurement of chicken feet gelatine (CFG) gels

Chicken feet were purchased in Raciola Uherský Brod, Czech Republic, and processed to chicken feet gelatine according to the method described by **Mokrejš et al. (2019)**. Two types of pork gelatines and two types of beef gelatines were purchased as well and used for the comparison.

Table 1 Initial Bloom values of chicken feet, beef and pork gelatine gels.

Type of gelatine	Beef266	Beef273	Pork212	Pork288	CFG240
Bloom value ±SD	266 ±3	273 ±2	212 ±2	288 ±4	240 ±3

Note: CFG denote chicken feet gelatine; Beef266 and Beef273 are beef gelatines with Bloom values of 266 and 273; Pork212 and pork288 are pork gelatines with Bloom values of 212 and 288.

Gelatine gels were prepared following **GMIA – Standard Testing Methods for Edible Gelatin (2019)** so that 7.5 g of gelatine was mixed with 105 mL distilled water and placed into a standardized bloom jar with a volume of 150 mL and dimensions as follows: overall height of 85 mm, inside diameter of 59 mm, outside diameter of 66 mm, neck inside diameter of 41 mm, and a shoulder height of 65 mm. The mixture was allowed to swell at room temperature for 1 – 3 h. Afterward, the bloom jar (Figure 1) was heated in a water bath at 65 °C to prepare a gelatine solution which was cooled at room temperature and maintained in a cooling box for 16 – 18 h to form gelatine gel. Gel strength (or Bloom value) was measured by Stevens LFRA Texture Analyser (Leonard Farnell and Co Ltd., England, Figure 1). Gelatine gel strength is defined as a force (weight in g) required to depress a measuring probe by specific penetration to a definite area of the gelatine surface to a particular distance.

Figure 1 Prepared chicken feet gelatine gels (left) Stevens LFRA Texture Analyser (right).

Determination of thermal stability of gelatine gels

Studies examining gelatine gels thermal stability mostly used methods of thermal analysis (DSC), rheological testing, or determination of activating energy. Considering a wide range of applications of gelatines in the food industry assessing the quality of gelatines based on their gels strength, this study has employed a method of measuring gel strength according to **GMIA - Standard Testing Methods for Edible Gelatin (2019)**. It has monitored a decline of gel strength in time which is in contrast with other studies that have not considered testing of gelatines on a long-term time scale. The thermal stability of gelatine gels was expressed as a percentage change (decline) of gelatine gel strength during the time period of 5 days. Gelatine gel strength was measured every hour within the first 8 hours of the experiment, and then after 16, 23, 87, 93, 111, and 120 hours. Experiments were performed at three different temperatures of 23, 29, and 35 °C with the relative humidity of 60 and 80%. In total, six series of experiments were performed in which 30 gelatine samples were analysed.

Statistic analysis

1-sample and 2-sample standard deviation tests on the significance level of $p = 0.05$ were applied to all results using Minitab 18 statistical software for Windows (Minitab, Ltd., USA). All analyses were performed in triplicate and arithmetic means and standard deviations were calculated.

RESULTS AND DISCUSSION

The gel strength of prepared chicken feet and commercial pork and beef gelatine gels

Table 1 displays initial Bloom values of commercial mammal and prepared chicken feet gelatines ranging in all samples between 220 and 300 which means all tested gelatines showed a significant gel strength **(Johnston-Bank, 1983)**. The highest gel strength was recorded in Pork288 and the lowest in Pork212. The gel strength of prepared chicken feet gelatine was 240 Bloom. Gel strength of commercial pork and beef gelatine gels ranging from 100 to 300 Bloom, whereas 200 – 250 Bloom is most preferred **(Holzer, 1996)**. Several studies discussing the preparation and testing of the strength of chicken feet gelatine gels were published. **Taufik et al. (2010)** examined the effect of age and extraction temperature on characteristics of chicken feet skin gelatine and reported Bloom values from 112 to 125 which is significantly lower than the values of chicken feet gelatine prepared in this study. **Rahman and Jamalulail (2012)** performed extractions of chicken feet gelatine, inspected its physicochemical characteristics and sensory quality, and recorded the value of 264 Bloom which is slightly higher than the value of chicken feet gelatine established in this study. **Widyasari and Rawdkuen (2014)** described gelatine obtained from chicken feet by acid and ultrasound-assisted extraction and reported Bloom values of 185 and 79 which is less than this study provided. **Chakka et al. (2016)** extracted chicken feet gelatine using food-grade acids and monitored the gel strength in the range from 119 to 204 Bloom which is less than it was recorded in this study. **Almeida and Lannes (2013)** extracted gelatine from chicken feet and characterized its physicochemical properties. They confirmed the gel strength of 295 Bloom which is more than it was measured in this study. **Sompie and Triasih (2018)** reported very low gel strength of chicken leg skin gelatine (78 Bloom). On the contrary, in the case of gelatine extracted from residues after mechanical processing of poultry meat, very high Bloom values were recorded: 309-318 Bloom **(Fonkwe and Singh, 1997)** and 374-380 Bloom **(Rafieian et al., 2013)** and **(Rafieian et al., 2015)**. High Bloom values have also been reported for poultry gelatine by other researchers: 294 Bloom **(Almeida, Calarge and**

Santana, 2013), 338 Bloom **(Du, et al., 2014)** and 355 Bloom **(Ee et al., 2019)** and **(Sarbon et al., 2013)**. Such differences in results may stem from different extraction conditions, particularly time and temperature, as well as from the applied pre-treatment method. The age and gender of the animal could also affect the quality of prepared gelatines.

Determination of thermal stability of prepared chicken feet and commercial pork and beef gelatine gels

The results of determination of the thermal stability of chicken feet, beef, and pork gelatine gels tested at the temperatures of 23, 29, and 35 °C and relative humidity of 60 and 80% are shown in Tables 2 – 7. Initial Bloom values of gelatines are expressed as 100%. Changes in gelatine gel strength (declines) are expressed as Bloom index in %.

Table 2 shows the results of the thermal stability of chicken feet, pork, and beef gelatines at a temperature of 23 °C and humidity of 60%. Gel strength gradually decreased as expected. After one hour of measurement, the slightest decline was recorded in CFG240 and the highest in Beef273. A drop of gel strength in other types of gelatines was approximately 25%; differences were not statistically significant (p >0.05). Similar trends were observed also in the following measurements with declines of gel strength between 30 and 65%. The smallest decrease of gel strength was recorded in chicken feet gelatine while the most significant drop was determined in Beef273 (3 – 5 h of measurement) followed by Pork212 (87 – 120 h of measurement). Within the last two measurements (after 111 and 120 h), no further changes in gel strength were established. The deepest decline in gel strength in the final measurement was monitored in Beef266 (more than 90%) while the smallest drop was identified in prepared gelatine CFG240 (approximately 75%); this gelatine showed this trend for the whole testing period. The reduction of gel strength in other types of gelatines was approximately 85% with statistically insignificant differences (p >0.05).

Table 3 summarizes the measurements of the thermal stability of chicken feet, pork and beef gelatines at the temperature of 23 °C and humidity of 80%. Prepared chicken feet gelatine CFG240 provided values with statistically insignificant differences (p >0.05) recorded at both levels of humidity – 60 and 80%.

Table 2 Thermal stability of chicken feet, beef and pork gelatines at 23 °C and relative humidity of 60%.

Temperature of 23 °C; relative humidity of 60%	Type of gelatine/Bloom index (%)				
Time of measurement (h)	Beef266	Beef273	Pork212	Pork288	CFG240
0	100	100	100	100	100
1	77	61	76	75	87
2	50	35	47	50	68
3	35	27	33	37	57
4	28	26	24	29	51
5	24	25	21	26	49
6	23	24	20	25	45
7	22	23	19	24	45
8	22	22	18	23	44
16	21	21	18	20	40
23	20	20	17	18	39
87	8	14	15	16	28
93	8	13	14	16	24
111	8	13	14	15	23
120	8	13	14	15	23

Table 3 Thermal stability of chicken feet, beef and pork gelatines at 23 °C and relative humidity of 80%.

Temperature of 23 °C; relative humidity of 80%	Type of gelatine/Bloom index (%)				
Time of measurement (h)	Beef266	Beef273	Pork212	Pork288	CFG240
0	100	100	100	100	100
1	82	70	82	87	85
2	58	46	56	62	67
3	45	37	42	49	57
4	37	32	34	41	50
5	34	31	30	37	48
6	32	31	25	35	46
7	31	31	24	33	44
8	30	30	24	32	43
16	29	29	24	32	40
23	29	24	24	32	38
87	19	20	18	22	27
93	19	20	18	22	25
111	19	20	18	22	23
120	19	20	18	22	23

However, within commercial gelatines in the first 6 hours of measurement, higher declines of gelatine gel strength with statistically significant differences (p <0.05) were recorded at the humidity of 80% than 60 %. In the following measurements, statistically significant (p <0.05) smaller declines of commercial gelatine gel strength were recorded at the humidity of 80% than 60%. After 1 h of measurement, the slightest drop of gelatine gel strength was identified in Pork288 and the most considerable in Beef273. In all the following measurement CFG240 showed the smallest decline. After two hours of testing, the fall of gelatine gel strength ranged from 33 to 64%. The deepest decline in 2, 3, and 4 h of measurement were recorded in Beef273 whereas in further measurements the most significant decrease was determined in Pork212. Nevertheless, within the measurement in 111 and 120 h, no further changes in gelatine gel strength were identified with the final declines of the strength of approximately 80%. Prepared chicken feet gelatine performed either comparable or even better thermal stability at a humidity of 60 and 80% if compared with commercial beef and pork gelatine.

The results of the thermal stability of chicken feet, pork, and beef gelatines at the temperature of 29 °C and humidity of 60% are displayed in Table 4. In contrast with the measurements at the temperature of 23 °C and humidity of 60%, a significantly deeper (p <0.05) decline of gel strength in all gelatines was monitored in 2-h measurement and further. After 1 h, the smallest decrease was monitored in Beef266 and both pork gelatines, while in CFG240 and Beef273 the deepest reduction of gel strength was recorded. In further measurements, the smallest drop was identified in Beef266 and Pork288 (63%), slightly deeper in Pork212 and CFG240 (65%), and the most significant decline in Beef273 (72%). After 3 and 4 h of measurement, the smallest decrease was recorded in Beef266, Pork288, and CFG240 and the deepest fall in Beef273 and Pork212. After 5, 6, 7, and 8 h, gelatine CFG240 showed the slightest decline of approximately 90% while other types of gelatines performed slightly higher, yet statistically insignificant (p >0.05) declines of gelatine gel strength. A similar trend was observed in further measurements and after 87 h of measurement, no gelatine showed a change in gel strength. The final decline of gel strength was almost 100%.

Table 4 Thermal stability of chicken feet, beef and pork gelatines at 29 °C and relative humidity of 60%.

Temperature of 29 °C; relative humidity of 60%	Type of gelatine/Bloom index (%)				
Time of measurement (h)	Beef266	Beef273	Pork212	Pork288	CFG240
0	100	100	100	100	100
1	76	63	77	75	66
2	37	28	33	37	33
3	21	16	16	21	20
4	14	12	11	14	15
5	11	10	8	10	13
6	9	9	7	9	11
7	8	8	6	8	9
8	7	7	5	6	7
16	4	5	4	4	6
23	4	4	3	4	5
87	3	4	3	3	3
93	3	4	3	3	3
111	3	4	3	3	3
120	3	4	3	3	3

Table 5 Thermal stability of chicken feet, beef and pork gelatines at 29 °C and relative humidity of 80%.

Temperature of 29 °C; relative humidity of 80%	Type of gelatine/Bloom index (%)				
Time of measurement (h)	Beef266	Beef273	Pork212	Pork288	CFG240
0	100	100	100	100	100
1	74	64	71	69	64
2	30	22	26	31	33
3	14	12	11	14	19
4	8	8	6	8	15
5	6	7	4	6	13
6	5	6	3	5	11
7	4	5	3	4	9
8	3	4	2	3	8
16	2	3	2	2	6
23	2	3	2	2	6
87	2	3	2	2	4
93	2	3	2	2	4
111	2	3	2	2	4
120	2	3	2	2	4

Table 5 shows the results of the thermal stability of chicken feet, pork, and beef gelatines at a temperature of 29 °C and humidity of 80%.

At this humidity, a slightly higher decline of gel strength was monitored in commercial gelatines than at the humidity of 60%; while in chicken feet gelatine no statistically significant differences (p >0.05) were recorded, similarly as at the temperature of 23 °C. After 1 h of measurement, the smallest decline was recorded in Beef266, slightly higher in pork gelatines and the deepest decrease in Beef273 a CFG240. In contrast, after 2 h of measurement, the smallest drop of approximately 70% was in CFG240, slightly deeper in Beef266 and Pork288, and the most significant reduction of approximately 80% in Beef273. After 3 h, the last drop was in CFG240, similarly to further measurements, while commercial gelatines performed slightly deeper reduction of gel strength. Further measurements showed a trend of a gradual decline of gel strength; importantly, commercial gelatines performed statistically significant (p <0.05) higher decrease of gel strength if compared with chicken feet gelatine. After 87 h, almost 100% declines in gel strength in all gelatines were monitored. It has been proved that chicken gelatine performs comparable or even better properties than commercial gelatines considering the thermal stability of gelatine gel strength.

Table 6 summarizes the results of the thermal stability of chicken feet, pork, and beef gelatines at the temperature of 35 °C and humidity of 60%. All gelatines performed a steep decline of gelatine gel strength at this temperature. After 1 h of measurement, the decrease ranged between 38 and 43% with the smallest drop in CFG240 and the highest in Beef266 and Pork212. A dramatic decline of gel strength from 83 to 90% was recorded in further measurements. The smallest reduction was determined in Beef266 and the highest in Beef273. After 3 h of measurement, the smallest decline of more than 90% was in CFG240 while all commercial gelatines showed at a drop of 97%. After 4 h, almost all gelatines performed a decrease of nearly 100% and no gelatine formed a gel after 5 h of measurement.

The results of the thermal stability of chicken feet, pork, and beef gelatines at the temperature of 35 °C and humidity of 80% are shown in Table 7. The figures do not differ much from the results at the same temperature at the humidity of 60%. After 1 h of measurement, the decline of gel strength varied from 36 to 42% which proves no statistically significant differences (p >0.05) between the experiments at the humidity of 80 and 60%. The smallest decrease of gel strength was established in CFG240 and Pork288 while the deepest drop was recorded in Beef266, equally as at the humidity of 60%. After 2 h of measurement, chicken feet gelatine showed the smallest decline (75%), and Pork212 together with other beef gelatines the biggest fall (85%). After 3 h, CFG240 performed a decline of gel strength of more than 91% and commercial gelatines of 97%. Nearly 100% decline of gel strength in all gelatines was recorded after 4 h of measurement. Equally, as in previous experiments, comparable declines of gelatine gel strength were recorded in both chicken feet and commercial gelatines at the temperature of 35 °C.

CONCLUSION

Chicken feet gelatine (CFG) was obtained from chicken feet, a slaughterhouse by-product, using a biotechnological method with a previous proteolytic enzyme pre-treatment and extracted at the temperature of 80 °C for 45 min. Samples of gels were prepared both from CFG and commercial pork and beef gelatine and their thermal stability were tested. For the experiments, the thermal stability of gelatine gels was defined as a percentage change of gel strength in time and it was recorded at temperatures of 23, 29, and 35 °C at the relative humidity of 60 and 80% for 5 days. These temperatures and humidities were proposed to reflect the climatic conditions common during the storage of products containing gelatine gel in the summer or tropical areas. The results have shown that the thermal stability of gelatine gels is lower at higher storage temperatures confirming the hypothesis. However, the assumption that higher humidity will cause a steeper decline in gel strength has not been proved.

Table 6 Thermal stability of chicken feet, beef and pork gelatines at 35 °C and relative humidity of 60%.

Temperature of 35 °C; relative humidity of 60%	Type of gelatine/Bloom index (%)				
Time of measurement (h)	Beef266	Beef273	Pork212	Pork288	CFG240
0	100	100	100	100	100
1	57	58	57	61	62
2	17	11	15	12	15
3	3	3	3	3	7
4	2	2	1	2	3
5	0	0	0	0	0

Table 7 Thermal stability of chicken feet, beef and pork gelatines at 35 °C and relative humidity of 80%.

Temperature of 35 °C; relative humidity of 80%	Type of gelatine/Bloom index (%)				
Time of measurement (h)	Beef266	Beef273	Pork212	Pork288	CFG240
0	100	100	100	100	100
1	58	60	60	64	64
2	15	15	15	19	25
3	3	3	3	3	9
4	2	2	2	2	3
5	0	0	0	0	0

The thermal stability of prepared chicken feet gelatine has not been significantly affected by humidity. On the other hand, the results of commercial gelatines at different humidities were not definite.

This fact favours the applications of chicken gelatine in the food industry; the thermal stability of chicken gelatine was higher or similar to the stability of commercial mammalian gelatines. Experiments at a temperature of 23 °C showed that the highest thermal stability was recorded in chicken gelatine at the humidity of both 60 and 80%. The decline of the strength of chicken feet gelatine gel was in earlier times of measurement approximately 1.5x lower and in later times of measurements even nearly as 2x lower than in commercial gelatines. At the temperature of 29 °C, comparable results in both commercial and chicken feet gelatines were established. The last tested temperature was 35 °C also providing a similar decline of the strength in chicken feet gelatine gels and commercial gelatines. This study has confirmed that laboratory prepared chicken feet gelatine provides similar thermal stability to gels of commercial pork and beef gelatine. Hence chicken feet gelatine may be employed as a potential alternative to traditional gelatines used in the food industry in the production of confections, aspics, or desserts. This data clarifying the behavior of gelatine gels thermal stability is beneficial also for further industrial sectors, such as for the preparation of hard gelatine capsules in the pharmaceutical industry.

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DOES FOODSERVICE INDUSTRY CARE ABOUT CSR? A STUDY IN PORTUGAL AND UKRAINE

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ABSTRACT

The purpose of the article is to compare the CSR perception level of the food service industry in Portugal and Ukraine. The main goal of the study is to find the attitudes in Portugal and in Ukraine towards CSR. Additionally, two hypotheses were formulated to understand the possible relation between (1) the financial situation and (2) the duration of the company with the awareness level about the CSR concept. A quantitative approach, using questionnaires, was adopted to survey using a sample of 201 representatives of SMEs in the foodservice sector (101 Portuguese and 100 Ukrainian), in 2019. Descriptive statistics and Spearman test were used to analyze the data. The present work demonstrates a significant correlation between the financial situation of the company and the CSR awareness level of leadership. Furthermore, the research shows that activities related to the economic sphere and CSR workplace policy among food sector companies in Ukraine and Portugal are developed to a great extent. From this point of view, we can state, that the publication of the article is one of the results and supported by grant project KEGA 005SPU-4/2019 "Theory and Practice of the International Management and Entrepreneurship in Multicultural Environment". In this study, two different countries were chosen (Portugal and Ukraine), which are different in culture, history, and economic background. The paper focused on the food service industry and contributes to the ongoing debate about the SMEs and its involvement with CSR. It also helps schools to identify plans in CSR policy and topics for future research.

Keywords: Corporate Social Responsibility; Food service Industry; SMEs; Portugal; Ukraine

INTRODUCTION

The social orientation of the world economy requires the development of Corporate Social Responsibility (CSR) activities to ensure the free entry of domestic companies into world markets **(Van Marrewijk, 2003)** and to increase cooperation with foreign investors, maintaining a worthy competitive position in the international arena **(Secchi, 2007; Lee, 2008)**. The understanding of CSR importance pushes business to assume not only the behavior of law-abidingness but also to carry out voluntary participation in the implementation of corporate contribution to the development of the community, the territory, and establishing social partnership relations with the state **(Burja and Mihalache, 2010)**.

Although there has been an increase in CSR interest at a global scale trying to define CSR is not an easy task **(Nyarku and Ayekple, 2019; Aßländer, 2011)**. First of all, there are different economic traditions in Europe and the US, which results in different interpretations of corporations´ responsibilities. The American perspective of the concept is mainly oriented on ethical responsibility and corporate philanthropy (besides economic and legal obligations), while the European perspective sees such responsibilities as the task of government **(Kasych, 2014)**. As defined in **ISO 26000 (2010)**, social responsibility is the responsibility of an enterprise for the impact of its decisions and actions on society, the environment, through transparent and ethical behavior. Moreover, it is necessary to increase the number of studies related to CSR and SMEs **(Sweeney, 2007)**. Companies often lack a clear understanding and conscious recognition that CSR is not something exceptional, caused by special circumstances, but a norm that stems from the very essence of business, the philosophy of management **(2016; Kasych, 2014; Kravchenko, 2013)**. Furthermore, considering the new trend in the literature of describing the relationship between CSR and geography **(John et al., 2011; Loughran and Schultz, 2005; Sorenson and Baum, 2003)**, one can agree with the importance of showing the influence of company's geographical position on the process of CSR strategy formation. Thus, comparative researches on CSR development are needed not only within the neighboring countries but also within the countries which are located in different geographic areas **(Branco and Delgado, 2011)**.

Thus, this study aims to compare the CSR perception level in the foodservice sector in Portugal and Ukraine. Given the different geographic location and business environment, both countries, Ukraine and Portugal, are ideally suited for the study. It is also worth mentioning that the selected countries have a different cultural, historical, and economic background, which can affect the level of the CSR perception. However, the dynamic development of the food service market in Ukraine and Portugal is a common feature of the studied countries. For example, from the end of 2015 – beginning of 2016, the Portuguese public foodservice market has started to emerge from the crisis, driven by the growth of the tourism industry **(GIRA Foodservice, 2017)**. During the last years, the Ukrainian food service market is as well trying to recover after the political and economic crisis of 2014 – 2015. There is an increase in the competition between food establishments, the emergence of new formats, and the use of innovative approaches in the activities of food service enterprises of the country **(Hirnyak and Glagola, 2018)**. The food service market was chosen as the main field of our research based on the before-mentioned facts. Specifically, the main goals of the study are to find the attitudes in Portugal and Ukraine towards CSR: identifying what does motivate food service SMEs to implement the instruments of CSR; what are the main hindrances that food service SMEs face to implement the CSR principles into their business strategies; and what kind of CSR-related activities are commonly carried out by the food service companies in environmental, economic and social pillars of the triple bottom line approach. Additionally, two hypotheses were formulated to understand the possible relation between (1) the financial situation and (2) the duration of the company with the awareness level about the CSR concept.

The rest of the paper is structured as follows. The second section reviews the literature on the context of CSR, including sub-sections to describe the current CSR development in Portugal and Ukraine. The hypotheses are presented in the third section, followed by the methodological issues in terms of the research design and sample identification included in the fourth section. The results are presented in the fifth and are discussed in the sixth section, followed by the conclusion and limitations along with suggestions for further research avenues.

CSR and the specific cases of CSR in Portugal and Ukraine

Nowadays, the development of a CSR strategy for an enterprise is one of the crucial components for its success. The implementation of CSR principles into company activities leads to a higher level of interconnection with the society. Moreover, it is necessary to underline that CSR is one of the most important factors on the way to economic development for an enterprise, as well as for the country **(Ebner and Baumgartner, 2006)** and contributes to sustainable development **(Behringer and Szegedi, 2016; Kolk and Van Tulder, 2010; Aras and Crowther, 2008)**.

Even though the concept of CSR has emerged within the last century, there is no universal definition of CSR **(Duman, Giritli and McDermott, 2016; Ness, 1992)**. There are already several models in understanding the concept of CSR, like the models of **Carroll (1979)**, **Wartick and Cochran (1985)**, **Wood (1991)**, or **Clarkson (1995)**, the study of **Elkington (1999)** on the Triple Bottom Line (TBL), measures the three pillars of CSR, economic, environmental and social performance of the organization **(Elkington, 1999)**. The three Ps: (i) profit (i.e., financial), (ii) people (i.e., social), and (iii) the planet (i.e., the environment) are still perceived as synonymous of CSR **(Fauzi, Svensson and Rahman, 2010; Rubenstein, 2003)**.

CSR is being widely developed in the European Union, where companies make voluntary commitments to labor protection, waste management, business ethics while presenting similar requirements to their foreign units and partners **(Marushchak, 2012)**. Portugal, being a member of the European Union, is promoting the development of CSR initiatives, leading to responsible business behaviour, improving the situation on the labour market, the quality of products and services provided by companies, as well as sustaining the development of the country as a whole. CSR in Portugal is determined at a national level and is a policy component of the different ministries **(Shevchenko, 2014)**.

Recent studies using Portuguese samples and context showed that employees who are aware of their company's CSR investment activities tend to make an additional effort in their work and even agree with the fact that CSR practices can lead to more effective company performance **(Story and Neves, 2015)**, for Portuguese consumers environmental CSR practices are much more important than social activities and practices **Loureiro et al. (2012)**. Thus, Portuguese scholars are making a huge research effort into the CSR field. However, there are still great opportunities for expanding and promoting the development of broader and more detailed research in this field and to explore different geographic contexts **(Branco and Delgado, 2011)**.

The Ukrainian scientific literature has been increasing the number of publications related to the CSR sphere annually **(Kasych and Yakovenko, 2014; Kostyrko, 2014)**, despite, in Ukraine, social responsibility is at an early stage of development **(Kolyanko and Myronov, 2016)**. As a result, domestic enterprises often take foreign companies' experience and practice as an example when trying to implement a CSR strategy. The main obstacles to the development and promotion of social responsibility are lack of funds, high tax burden, lack of interest in social responsibility has been suggested in the study of **Burkovska and Lunkina (2016)**. The main trends, barriers, and prospects for CSR developing in Ukraine has been explored as well **(Zinchenko and Saprykina, 2017)**, recommending several steps for the future development in the CSR field, such as the development of a regulatory framework that would facilitate CSR activity, raising the awareness of the National Contact Center for Responsible Business Behavior, implementation of CSR courses into university programs, among others.

Scientific hypothesis

Social responsibility of business should be considered not only as a way of social stabilization but as an instrument for improving the financial situation of the company. However, the relationship between CSR and the company's financial situation can be positive, negative, or

neutral. Some studies have concluded a positive correlation between CSR and the company's financial performance **(Fauzi and Idris, 2009; Orlitzky and Benjamin, 2001; Simpson and Kohers, 2002; Yefimenko et al., 2014)**, others show a negative relationship **(Patten, 2002)** and some neutral relationship as well **(McWilliams and Siegel, 2001; Moore, 2001)**.

Thus, the following hypothesis is suggested:

Hypothesis 1: There is a significant correlation between the financial situation of the company and the awareness level about the CSR concept. The duration of the company's existence affects the level of CSR development among its' business units **(Okhrimenko and Ivanova, 2015)**. Researchers believe that companies that are operating for a long time have more experience in the business environment and therefore better understand how to properly implement and use the CSR method. Consequently, newly formed companies demonstrate the lack of CSR management experience and skills. Mostly it happens because business owners do not have a clear understanding of the CSR in the process of strategic development. Moreover, the lack of fast, obvious results from the implementation of the CSR strategy is the barrier of CSR activities development among newly formed companies **(Sardak and Shmyhovska, 2017)**. Thus, the following hypothesis is suggested:

Hypothesis 2: There is a significant correlation between the duration of the company's existence and the awareness level about the CSR concept.

MATERIAL AND METHODOLOGY

In the present study, it is compared to the development of CSR activities among Portugal a Ukraine food service companies. The objective to identify what does motivate food service SMEs to implement the instruments of CSR, what are the main hindrances that food service SMEs face to implement the CSR principles into their business strategies, and what kind of CSR related activities are commonly carried out by the food service companies in environmental, economic and social pillars of the triple bottom line approach. Additionally, who hypotheses were formulated to understand the possible relation between (1) the financial situation and (2) the duration of the company with the awareness level about the CSR concept, as presented in the previous section.

The research was conducted among the companies of the food sector of Portugal and Ukraine operating in three cities of each country (Portugal: Lisbon, Porto, Faro; Ukraine: Kyiv, Lviv, Odesa). The selection can be explained by the fact that according to Statistics Portugal – Web Portal **(INE, 2018)** and State Statistics Service of Ukraine **(UKRSTAT, 2018)**, the before-mentioned cities are the most visited ones. A questionnaire method was used to collect data in January – February 2019 (Ukraine) and March- April 2019 (Portugal). The questionnaire forms were fulfilled by representatives of executive positions. The communication with the respondents was conducted by sending out an online sample of the questionnaire via e-mail and, in some cases, through personal meetings with the respondents. The questionnaire was developed from a thorough literature review **(Zinchenko and Saprykina, 2017)**, which consisted of 15 questions grouped into 6 parts. The questionnaire includes closed and semi-closed questions:

- Part I: represents formal questions about company activities, such as financial status, the duration of the firm's existence, company size;
- Part II: includes questions that allow estimating level of leadership awareness of CSR notion. This part of the questionnaire allows us to find out factors which hinder CSR implementation and factors which, on the other hand, encourage companies to implement and develop activities in this area;
- Part III, IV and V: allow to evaluate the company's activities in three main CSR pillars: economic pillar; environmental pillar and social pillar;
- Part VI: includes questions regarding the company's plans for further CSR development activities.

The questionnaire adopted Likert scales, in which the respondent expressed the extent of his/her agreement or disagreement with a certain statement, ranging from 1 – "strongly disagree" to 5 – "strongly agree" or 1 – "not dealing with" to 5 – "dealing a lot". To analyze the data, Excel and XLSTAT version 2019.1.3 were used. In order to test the hypotheses, the Spearman correlation coefficient was used.

Statistical analysis

A total of 201 responses were collected: 101 (in Portugal) and 100 (in Ukraine), from representatives of executive positions in Portuguese and Ukrainian companies. Concerning the Portuguese sample companies, the majority was formed by the small-sized enterprises (80.2%) and medium-sized (19.8%). The company's existence duration was the following: 8.9% less than 1 year, 24.8% from 1 to 5 years, 51.5% from 5 to 10 years and 14.9% more than 10 years. The majority (88.1%) has a domestic or local owner and 11.9% of the respondents have a domestic owner with a foreign investor. 31% of respondents declared to have a very good financial situation, 32.7% – above average, 22.8 % – average and 12.9% – below average. In the case of 100 Ukrainian enterprises, most of them are small-sized (60%), and the medium-sized companies (40%). The company's existence duration was the following: 5% less than 1 year, 28% from 1 to 5 years, 47% from 5 to 10 years and 20% more than 10 years. 79% of respondents declared to have a domestic owner, 21% have a domestic owner with a foreign investor. The majority (40%) has a very good financial situation, 22% – above average, 20% – average, and 18% – below average.

RESULTS AND DISCUSSION

The first questions were aimed to explore the general attitude of the respondents toward CSR. 63.4% of Portuguese and 61% Ukrainian respondents declared that they are familiar with the term of CSR. Consequently, it can be stated that the CSR concept is well known for most of the enterprises in the investigated market.

The Likert scale helped us with further investigation of the food service companies. Table 1 explicitly shows the extent to which companies agree or disagree with the following statement: "The representatives of executive positions have a good knowledge of CSR concept and the

ways of CSR implementation into company's activities". Based on the results, only 16.8% of Portuguese and 19% of Ukrainian respondents strongly agree that the company's leadership has a good knowledge of the CSR concept. However, it is worth mentioning that 48.5% of Portuguese and 35% of Ukrainian respondents see themselves as a socially responsible enterprise.

Table 2 shows that regardless of the country, almost all respondents indicate moral-ethical reasons (97% of Portuguese and Ukrainian respondents), improvement of economic indicators (96% of Portuguese and Ukrainian respondents), maintaining/increasing of the company´s reputation (98% of Portuguese and 100% of Ukrainian respondents), increasing of the employee motivation (98% of Portuguese and 97% of Ukrainian respondents), increasing/maintaining the level of customers' loyalty (99% of Portuguese and 95% of Ukrainian respondents) as the main motivating factors for CSR implementation. It is also worth noting that third-party pressures do not motivate enterprises to implement CSR activities in their businesses.

A weak CSR legislation is one of the main barriers to implement/improve CSR activities among Ukrainian food service companies (78% of respondents). In turn, the majority of Portuguese respondents (75.2%) noted that for them the lack of time is one of the biggest obstacles for CSR implementation. The positive fact is that only a few firms have mentioned that CSR activities are not important for their company (5% of Ukrainian and 4% of Portuguese respondents) (Table 3).

Economic Pillar

Table 5 shows that most of the respondents (851% of Portuguese and 85% of Ukrainian companies) implement principles of fair trade while doing their business. Moreover, the majority tends to provide information to the stakeholders (consumers, suppliers) (64.4% of Portuguese and 73% of Ukrainian companies) and tends to carry out transparent activities (62.4% of Portuguese and 72% of Ukrainian companies). From the other side, considering the ethical principles of trade the sales business innovations related activities are not present among the foodservice companies' activities.

Table 1 Respondents' agreement/disagreement with statements toward CSR.

Statement	Mark Country	1 – strongly disagree	2	3	4	5 – strongly agree
The representatives of executive positions have a good knowledge of the CSR concept and the ways of its implementation in the company's activity	Portugal	0%	3%	37.6%	42.6%	16.8%
	Ukraine	0%	9%	29.0%	43.0%	19.0%
Our company is a socially responsible company	Portugal	0%	0%	21.8%	29.7%	48.5%
	Ukraine	0%	1%	32.0%	32.0%	35.0%

Table 2 Factors that motivate/do not motivate a company to implement CSR.

Factors	Portugal		Ukraine	
	Do not motivate	Motivate	Do not motivate	Motivate
Moral-ethical reasons	3%	97%	3%	97%
The improving of economic indicators	4%	96%	4%	96%
Better relationships with investors	33.7%	66.3%	50%	50%
Better relationships with community	5%	9%	26%	74%
The maintaining/increasing the company´s reputation	2%	98%	0%	100%
The increasing employee motivation	2%	98%	3%	97%
The desire of the environment protection	21.8%	78.2%	35%	65%
Third party pressures (buyers, competitors, suppliers, etc.)	88.1%	11.9%	95%	5%
The increasing/maintaining the level of customers' loyalty	1	99%	5%	9%

Table 3 The main barriers to implement/improve company's CSR activities.

Factor	Country	
	Portugal	Ukraine
Shortage of knowledge	24.8%	47%
Resources shortage	39.6%	39%
Weak legislation dealing with CSR	2%	78%
Weak employees' motivation	1%	6%
Lack of human resources	39.6%	24%
Time shortage to implement	75.2%	43%
It is not important for the company	4%	5%

Table 4 The evaluation of the environmental pillar.

Activity	Mark Country	1 – not dealing with	2	3	4	5 – dealing a lot
Energy saving	Portugal	0%	27.7%	57.4%	14.9%	0%
	Ukraine	0%	23%	50%	27%	0%
Environmentally friendly work processes	Portugal	0%	5%	44.5%	50.5%	0%
	Ukraine	0%	2%	31%	54%	13%
Increase employees´ knowledge about environmental protection	Portugal	0%	20.8%	59.4%	19.8%	0%
	Ukraine	0%	27%	48%	22%	3%
Minimization of environmental impacts	Portugal	1%	1%	2%	30.7%	65.3%
	Ukraine	0%	0%	1%	41%	58%
Promoting cooperation with other companies in the environmental field	Portugal	26.7%	49.5%	22.8%	1%	0%
	Ukraine	27%	36%	35%	2%	0%
Purchase of environmentally friendly machinery and equipment	Portugal	0%	17.8%	66.3%	13.9%	2%
	Ukraine	0%	15%	36%	46%	3%
Waste sorting	Portugal	0%	0%	2%	31.7%	66.3%
	Ukraine	0%	0%	6%	35%	59%
Reducing material and energy-intensive processes	Portugal	0%	12.9%	68.3%	17.8%	1%
	Ukraine	0%	7%	41%	50%	2%
Research and development in the field of environmental protection	Portugal	60.4%	34.6%	5%	0%	0%
	Ukraine	59%	32%	9%	0%	0%
Transport optimization	Portugal	0%	1%	41.6%	50.5%	6.9%
	Ukraine	0%	7%	23%	60%	10%
Waste minimization	Portugal	0%	1%	41.6%	50.5%	6.9%
	Ukraine	0%	1%	16%	50%	33%
Water saving	Portugal	0%	0%	25.7%	53.5%	20.8%
	Ukraine	0%	0%	22%	54%	24%

Table 5 The evaluation of economic pillar.

Activity	Mark Country	1 – not dealing with	2	3	4	5 – dealing a lot
Ethical principles in trade	Portugal	0%	0%	1%	50.5%	48.5%
	Ukraine	0%	0%	4%	42%	54%
Fair trade	Portugal	0%	0%	0%	14.9%	85.1%
	Ukraine	0%	0%	0%	15%	85%
Innovations in sales business activities taking into account the ethical principles of trade	Portugal	52.4%	42.6%	3%	2%	0%
	Ukraine	26%	26%	40%	8%	0%
Processing of invoices on time	Portugal	2%	0%	0%	50.5%	47.5%
	Ukraine	0%	0%	1%	49%	50%
Providing benefits to disabled customers	Portugal	23.8%	37.6%	35.6%	2%	1%
	Ukraine	17%	23%	34%	26%	0%
Providing customer service after the sale of products and services	Portugal	0%	0%	5%	44.5%	50.5%
	Ukraine	0%	0%	2%	47%	51%
Providing information to stakeholders (consumers, suppliers, …)	Portugal	0%	0%	1%	34.6%	64.4%
	Ukraine	0%	0%	0%	27%	73%
Solving complaints with shareholders, suppliers and business partners	Portugal	0%	0%	16.8%	37.6%	45.6%
	Ukraine	0%	1%	10%	53%	36%
Transparency of company´s activities	Portugal	0%	0%	0%	37.6%	62.4%
	Ukraine	0%	0%	2%	26%	72%

Social Pillar

Our study results show that most of the workplace policy activities listed in the questionnaire are being implemented to a high extent (Table 6). Regarding the overall view of the workplace policy (social pillar), respondents, regardless of the location, are trying to implement a considerable number of such activities: compliance with labour standards (Portuguese respondents – 95%, Ukrainian respondents – 95%); respect for human rights and freedoms (Portuguese respondents – 73.3%, Ukrainian respondents – 88%); safety and protection of health at the workplace (Portuguese respondents – 80%, Ukrainian respondents – 93%).

Another area of the social pillar (policy towards community) has a paradoxically low development level. Ukrainian companies cooperate with the public community only through non-financial contributions supporting the local community (38%). On the other hand, Portuguese companies support the community by helping in the organization of different events (33.7%) (Table 7).

Concluding Questions About CSR

The basic aim of this part is to examine the plans in CSR policy of the investigated companies.

The vast majority of the companies (79.2% of Portuguese and 91% of Ukrainian) is planning to increase CSR related activities in the next 3 years. It should be noted that none of the respondents expressed a desire to reduce the number of CSR related measures.

Testing the hypotheses

Analyzing the correlation between the financial situation of the company and the CSR concept awareness level among Portuguese and Ukrainian foodservice companies,

Table 6 The evaluation of the workplace policy (social pillar).

Activity	Mark Country	1 – not dealing with	2	3	4	5 – dealing a lot
Anti-corruption and bribery standards	Portugal	0%	0%	10.9%	63.4%	25.7%
	Ukraine	0%	0%	13%	57%	30%
Awareness of employees about important matters relating to the company	Portugal	0%	1%	33.7%	54.4%	10.9%
	Ukraine	0%	0%	26%	59%	15%
Compliance with labour standards	Portugal	0%	0%	0%	5%	95%
	Ukraine	0%	0%	0%	8%	92%
Development of qualification, skills, and long-lasting career of its employees	Portugal	0%	0%	37.6%	37.6%	24.8%
	Ukraine	0%	1%	30%	42%	27%
Employee loyalty to the company	Portugal	0%	5%	43.6%	35.6%	15.8%
	Ukraine	0%	1%	47%	47%	5%
Fair wage	Portugal	0%	0%	0%	33.7%	66.3%
	Ukraine	0%	0%	0%	39%	61%
Helping workers and their families	Portugal	1%	33.7%	56.4%	8.9%	0%
	Ukraine	1%	21%	60%	18%	0%
Protection of an intellectual property	Portugal	0%	39.6%	59.4%	1%	0%
	Ukraine	1%	34%	64%	1%	0%
Protection of specific groups of employees (as disabled, …)	Portugal	5%	37.6%	55.4%	2%	0%
	Ukraine	1%	20%	64%	15%	0%
Providing space for mental hygiene (as rest, nutrition, regeneration area, …)	Portugal	0%	0%	9%	45.5%	45.5%
	Ukraine	0%	0%	1%	63%	36%
Respect for human rights and freedoms	Portugal	0%	0%	0%	26.7%	73.3%
	Ukraine	0%	0%	0%	12%	88%
Safety and protection of health in the workplace	Portugal	0%	0%	0%	20%	80%
	Ukraine	0%	0%	0%	7%	93%
Work benefits (e.g. Home office, additional insurance, …)	Portugal	0%	0%	20.8%	29.7%	49.5%
	Ukraine	0%	1%	22%	44%	33%
Work-life balance of employees	Portugal	0%	0%	14.8%	31.7%	53.5%
	Ukraine	0%	0%	11%	42%	47%

Table 7 The evaluation of the policy towards the community (social pillar).

Activity	Mark Country	1 – not dealing with	2	3	4	5 – dealing a lot
Participation and membership in various associations that support the development of the local community	Portugal	8%	38.6%	52.4%	1%	0%
	Ukraine	6%	27%	45%	21%	1%
Providing financial contributions to support the local community	Portugal	1%	40.6%	51.5%	6.9%	0%
	Ukraine	1%	38%	45%	15%	1%
Providing non-financial contributions to support the local community	Portugal	0%	7.9%	48.5%	38.6%	5%
	Ukraine	0%	3%	19%	40%	38%
Purchase of raw materials and resources from local businesses	Portugal	0%	5%	59.4%	35.6%	0%
	Ukraine	0%	7%	61%	26%	6%
Support to the children and youth education in the local community	Portugal	1%	17.8%	44.6%	30.7%	5.9%
	Ukraine	0%	1%	30%	41%	28%
Support to the local community after natural disasters	Portugal	6%	78.2%	14.8%	1%	0%
	Ukraine	17%	51%	30%	2%	0%
Supporting the community in the organization of events (cultural, sports, …)	Portugal	0%	2%	29.7%	34.6%	33.7%
	Ukraine	0%	5%	34%	33%	28%
Conducting a systematic questionnaire survey about the company's activity	Portugal	11.9%	25.7%	48.5%	12.9%	1%
	Ukraine	21%	32%	35%	12%	0%
Creating a job positions in the region	Portugal	2%	49.5%	47.5%	1%	0%
	Ukraine	5%	33%	60%	2%	0%

Table 8 Correlation matrix according to Spearman's correlation (H1).

Variables	Financial situation of the company in last three years	The level of CSR concept awareness
Portugal		
Financial situation of the company in last three years	1	0.788*
The level of CSR concept awareness	0.788*	1
Ukraine		
Financial situation of the company in last three years	1	0.855*
The level of CSR concept awareness	0.855*	1

Note: *<0.0001.

using the Spearman's correlation test, results are statistically significant correlated ($p = 0.001$, $p < 0.05$), for both Ukrainian and Portuguese samples. Regardless of the country, there is a significant positive relationship between the two variables (Table 8). Thus, it can be concluded that H1 is confirmed.

Results

In this study, we have compared the level of CSR perception in Portugal and Ukraine, aiming to explore the main factors influencing the CSR perception level among the foodservice companies in both countries. The results of the study show that despite the different economic, cultural conditions, and even different geographic position of Ukraine and Portugal, respondents from both countries demonstrate a similar CSR perception level and are implementing almost identical activities in relation to CSR, contrary to previous findings from the literature that assumed that the geographic position of the company influences CSR perception level **(Boeprasert, 2012; John et al., 2011; Loughran and Schultz, 2005; Sorenson and Baum, 2003)**.

The respondents' answers to "what are the main hindrances that foodservice SMEs face to implement the CSR principles into their business strategies?" help us to understand that Portuguese and Ukrainian respondents have different perceptions of the barriers on the way of implementing/improving CSR activities. Limited implementation time is the main barrier on the way of the CSR activities implementation among the majority of Portuguese respondents. On the other hand, Ukrainian respondents pointed out that weak CSR legislation and low awareness of CSR are the main barriers to CSR realization. These facts support previous literature that claimed that the lack of knowledge on CSR is the most significant barrier for CSR implementation/improving activities **(Kovach, 2013)**. Also, other authors are dealing with this problem from several sides like **Janošková and Csikósová (2019)** and **Halasi et al. (2019)**.

The respondents' answers "what does motivate foodservice SMEs to implement the instruments of CSR?", give a possibility to figure out which factors motivate/do not motivate companies to implement CSR policy. Almost all respondents indicated that moral-ethical reasons,

economic indicators improvement, better relationships with the community, maintaining/increasing of the company´s reputation, increasing employees' motivation, increasing/maintaining customers' loyalty level are the most common factors that motivate companies to implement CSR activities. It is also worth saying that third-party pressures do not motivate enterprises to implement CSR activities in their business. These results support previous literature findings that claimed that the possibility of economic indicators improvement is a strong motivator for companies in CSR implementation activities **(Simpson and Kohers, 2002; Yefimenko et al., 2014)**.

The respondents' answers to "what kind of CSR-related activities are commonly carried out by the food service companies?", show that minimization of the impact on the environment and waste sorting is the most common activity from the listed in the questionnaire. As for the economic pillar of CSR, the respondents show the high implementation level of almost all measures in this direction. This situation can be explained by the fact that such kind of activities help the companies to increase the profit, to improve financial and economic indicators, which is the main task for any enterprise. The study shows that companies have a well-developed policy at the workplace. Most respondents have a fair salary system, demonstrate compliance with labor standards, human rights, and freedom protection. Based on this we can sum up that companies understand that a successful business can be built only with the help of skilled workers who need to have a proper working condition. On the other hand, the social pillar (policy towards community) has a paradoxically low development level. In general, Portuguese and Ukrainian foodservice companies are usually engaged in the implementation of similar activities in all three CSR directions. This study also confirmed that enterprises choose activities that are low-priced or easy to implement **(Kolyanko and Myronov, 2016)**. Only measures that are regulated by law are implemented on a high level (such as fair trade, compliance with labor standards, etc.).

The present study claims that there is a significant correlation between the financial situation of the company and the awareness level of executive positions representatives about the CSR concept. Indeed, companies with a better financial situation demonstrate a higher level of awareness of the CSR concept and the ways of its implementation in the company's activities, corroborating our hypothesis 1. This conclusion supports previous literature findings that claimed a positive relationship between awareness of the importance of the CSR activities and the financial situation of the company **(Fauzi and Idris, 2009; Orlitzky and Benjamin, 2001; Simpson and Kohers, 2002; Yefimenko et al., 2014)**. On the other hand, results disprove the fact that the duration of the company's existence influences the company's leadership awareness of CSR concept **(Okhrimenko and Ivanova, 2015; Sardak and Shmyhovska, 2017)**, not corroborating our Hypothesis 2.

CONCLUSION

This study shows evidence that CSR practices in the environmental and social pillars are poorly developed compared to the other activities of the companies of our sample. Here the attention could be paid more to the privileges or so-called bonuses for companies that are trying to save energy, reduce the materials used, implement energy-intensive processes. Furthermore, the creation of the database on the local level that will clearly show regional requirements for social investments, promote social responsibility and help community development would be a great effective solution on the way of CSR implementation.

The empirical research findings of this study confirm a significant part of the literature review and help us to make important assumptions. The present work demonstrates a significant correlation between the financial situation of the company and the CSR awareness level of leadership. Furthermore, the research shows that activities related to the economic sphere and CSR workplace policy among food sector companies in Ukraine and Portugal are developed to a high extent.

Some limitations can be pointed out. Firstly, the sample is focused on one single industry, the food sector. Future studies could explore CSR perception and awareness in other business sectors with distinct features. Secondly, this study was conducted in Ukraine and Portugal only. Future research could focus on the CSR perception level of companies from countries located in different geographical areas, as outside of Europe. Thirdly, the questionnaire sample has a limited list of measures related to each of the CSR pillars. We suggest that future studies try to explore other CSR activities related to the environmental, economic and, social areas.

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ASSESSMENT OF POSSIBILITIES OF STRAWBERRY JAM REFORMULATION

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ABSTRACT

The prevalence of excessive weight gain, obesity, and associated diseases are permanently increasing. Therefore, the interest in food products with a composition suitable for people with the aforementioned health problems is also on the rise. The changes in food composition, nowadays often called reformulation, are mainly focused on reducing the amount of salt, sugar, or fat. Strawberry spreads with different sugar (10 – 40%) and strawberry (20 – 50%) content were prepared and the influence of strawberry jam composition on gel stiffness, colour, and sensory parameters was studied. This study aimed to determine the sensorial and technological limits (sugar and strawberry content) of strawberry jam reformulation. Carrageenan was chosen as a suitable gelling agent for the preparation of these reformulated strawberry products. strawberry spreads. The applicable concentration of carrageenan for the ideal stiffness of strawberry spreads was 2%. The results of the maximum compression force show a statistically significant increase of gel stiffness with increasing addition of strawberry puree, the effect of sugar content was also statistically significant (p = 0.05). This study showed that strawberry spreads with low strawberry and/or sugar content are sensorially acceptable.

Keywords: fruit spreads; carrageenan; texture characteristics; colour

INTRODUCTION

At the end of 2011, the world population had reached 7 billion and it is supposed the increase of one billion people to 2025. Around one billion people in the 21st century are starving and another billion people are suffering from micronutrient deficiency. On the other hand, over a billion people worldwide are deemed overweight or obese, with increased risk of associated diseases. Even with sufficient intake or excessive energy, consumed food can remain a poor source of essential nutrients. Opportunities to improve the nutritional profile and sustainability of the diet are found throughout the food chain, farm production, retail, and households. These include crop diversification, food enrichment, improvements in the efficiency of transport, waste minimisation, and last but not least food reformulation **(Buttriss, 2013)**.

The food industry has two main approaches to product reformulation: gradual reduction of nutrient content without introducing further changes in product formulation or partial/total replacement with other nutrients such as hydrocolloids, fat substitutes, and sweeteners **(Ares et al., 2018)**. However, some studies have found that products without added salt or sugars leave a negative sensory impression **(Phelps et al., 2006; DuBois and Prakash, 2012)**. Added sugars are defined as sugars that are added to foods during processing, preparation, or "on the table". They contribute to the energy value of the diet but have a little nutritional benefit and their high intake is associated with increased dietary energy, dental caries, and other adverse health effects such as excessive weight gain and reduced bone density. The current WHO recommendation is that the intake of added sugars should be less than 10% of the total energy intake **(WHO, 2015; Yeung et al., 2017)**. Replacing sugars in reformulated foods could represent a strategy to reduce sugar intake in the population without a significant change in the normal diet. However, reducing the sugar content of food is not easy because it results in changes to taste, texture, functionality and shelf life **(van Raaij, Hendriksen and Verhagen, 2009; Cruz et al., 2010)**. The hygroscopic nature of sugar plays an important role in reducing water activity in foods and helps maintain and prolong the shelf life of foods. Sugar prevents for example microbial spoilage of jams after opening. It is therefore always necessary to understand the function of sucrose in a particular food product before it is replaced **(Goldfein and Slavin, 2015)**.

Fruit, vegetables, and related products are often perceived by the consumer as "healthy" and consumable in any quantity. However, fruit and in particular for fruit products should be consumed with caution. Fruit products (compotes, fruit spreads, candied fruit, and other) often contain high sugar content. The effort to reformulate is then significant for the aforementioned reasons **(Rýdlová et al., 2019)**. The term "fruit spreads" is generally used in the food processing industry for marmalades, jams, jellies, and similar products. Fruit spreads have traditionally high

sugar content (60 – 65%), and there is the possibility for their reformulation. The formation of the desired jelly consistency of a fruit spread with reduced sugar content can be achieved by the use of low-esterified pectins or alternatively gelling agents based on gums or extracellular products of microorganisms **(Kadlec, Melzoch and Voldřich, 2012)**.

Scientific hypothesis

It is possible to reduce sugar content (up to 10%) and/or strawberry content (up to 20%) of the strawberry jam without it will result in less acceptable products.

There are technological and sensory acceptance limits for the sugar and/or strawberry content in strawberry spreads.

MATERIAL AND METHODOLOGY

Carrageenan C (CEAMGEL 9205; CEAMSA, Spain), citric acid (Hubka-Petrášek a vnuci s.r.o., Czech Republic), sucrose (DIAMANT; 1. Cukerní společnost Praha, s.r.o., Czech Republic), strawberry puree (100%) (Fruta Podivín, a.s., Hamé s.r.o., Czech Republic), distilled water, potassium hydrogen phthalate (SIGMA-ALDRICH, USA).

Sample preparation

Carrageenan gels (n = 2). 36 samples of carrageenan gels (C1 – C36, Table 1) with different sugar (10%, 20%, 30% and 40%), citric acid (0%, 0.25% and 0.5%) and carrageenan (1%, 1.5% and 2%) content were prepared. The calculated amount of sugar and carrageenan was added to boiling water in a multifunctional blender (Thermomix TM31, VORWERK, France), and the batch was agitated at 80 °C for 15 minutes. After 13 minutes of agitation, the calculated amount of citric acid was added (as a 50% water solution). The preparation of samples was carried out in the closed cooking blender. The total mass of one batch was 500 g. From each batch, 10 identical samples were prepared in 25 mL beakers. The cooked samples were stored in a refrigerator at 10 °C for 16 hours.

Strawberry spreads (n = 3). 16 samples of strawberry spreads (J1 – J16, Table 2, Figure 1) with different content of strawberry puree (20%, 30%, 40%, and 50%) and sugar (10%, 20%, 30%, and 40%) were prepared. Samples were prepared in the same way as carrageenan gels except that the strawberry puree was used instead of boiling water and heat-treated in a mixer at 90 °C for 10 min. The final mass of each batch was 500 g.

Methods

Gel stiffness (n = 10). Gel stiffness was analysed according to the method of **Sinthusamran, Benjakul and Kishimura (2014)** with a slight modification. The tests were performed with an Instron Model 5544 (Instron Ltd., USA). For the measurement, a 1-cm wide cylindrical plunger with a straight tip was used. The plunger's movement velocity was 80 $mm.min^{-1}$. The maximum force required to retract the plunger to a depth of 4 mm into the gel was recorded.

Titration acidity (n = 3**)** was measured by titration with 0.1 M NaOH (pH 8.1) and calculated as citric acid content (DL-22 Food & Beverage Analyzer, Mettler Toledo Co., Switzerland). Titration acidity was determined by **(AOAC, 2002)**.

Colour (n = 6). Colour determination was performed in the CIEL*a*b* (L^* lightness, a^* redness, b^* yellowness) colour space on a Minolta CM-5 spectrophotometer (Konica Minolta, Osaka, Japan). The parameters L^*, a^*, and b^* of strawberry spreads (J1 – J16) were determined **(Igual, Contreras, and Martínez-Navarrete, 2014)**. Samples were always dispensed into a cylindrical glass cell up to a height of 3 cm. The cell was placed on a measuring window with 30 mm diameters. Measurements were carried out in both the specular component included (SCI) mode and the specular component excluded (SCE) mode. The values measured in SCE mode were used for further processing in this study.

Soluble solid content (n = 3) of strawberry puree and strawberry spreads were measured using a digital refractometer (KRÜSS DR301-95, Germany).

Sensory analysis. The test room was equipped according to the requirements of the international standard **(UNI ISO 8589, 2007)**. The strawberry spread samples were evaluated by untrained sensory panellists (n = 30). The strawberry spread samples were evaluated for texture, sweet taste, sour taste, fruity taste, and overall acceptability. Sensory attributes were recorded using a nine-point hedonic scale (1 = dislike extremely; 5 = neither like nor dislike, 9 = like extremely).

Statistical analysis

Outliers, identified according to a Dean-Dixon test, were excluded at the significance level of $p = 0.05$. Results are presented as mean ± standard deviation. Data obtained from texture analysis, colour measurement, and sensory analysis were analysed using the Student t-test. Statistical analyses were carried out using the Statistica 12.0 software (Statsoft Inc., Tulsa, OK, USA). All the measurements of titration acidity and soluble solids were performed in triplicate. The measurement of colour was performed in six replicates, and the measurement of gel stiffness was performed ten times for each sample.

RESULTS AND DISCUSSION

According to the literature review **(Tang et al., 2019; Ellis et al., 2019; Ortiz-Tafoya et al., 2018)**, carrageenan was chosen as a gelling agent for reformulated strawberry jam. The model samples of carrageenan gel were prepared (Table 1), and gel stiffness was measured. The results of the carrageenan gel stiffness are shown in Figure 2, Figure 3, and Figure 4. The results show that carrageenan, sugar, and citric acid content influence the gelling ability and gel stiffness of carrageenan gels. The gel stiffness increased significantly with increasing amounts of carrageenan. Likewise, the addition of sugar slightly increased the gel stiffness, as confirmed by the results of studies by **Lopez-Sanchez et al. (2018)**, **Maurer, Junghans, and Vilgis (2012)**, **Deszczynski, Kasapis and Mitchell (2003)** and **Watase et al. (1990)**. However, the addition of citric acid significantly reduces the gel stiffness of carrageenan gels ($p = 0.05$).

Table 1 Recipes of carrageenan gels for evaluation of sugar and citric acid content on the gelling ability of carrageenan.

Content of citric acid = 0%		**Content of carrageenan C [%]**		
		1	1.5	2
Sugar content [%]	10	C1	C5	C9
	20	C2	C6	C10
	30	C3	C7	C11
	40	C4	C8	C12
Content of citric acid = 0.25%		**Content of carrageenan C [%]**		
		1	1.5	2
Sugar content [%]	10	C13	C17	C21
	20	C14	C18	C22
	30	C15	C19	C23
	40	C16	C20	C24
Content of citric acid = 0.5%		**Content of carrageenan C [%]**		
		1	1.5	2
Sugar content [%]	10	C25	C29	C33
	20	C26	C30	C34
	30	C27	C31	C35
	40	C28	C32	C36

Table 2 Recipes of strawberry spreads with carrageenan as the gelling agent.

Content of carrageenan C = 2%		**Content of strawberry puree [%]**			
		20	**30**	**40**	**50**
Sugar content [%]	**10**	J1	J5	J9	J13
	20	J2	J6	J10	J14
	30	J3	J7	J11	J15
	40	J4	J8	J12	J16
Addition of citric acid [%]		**0.32**	**0.23**	**0.14**	**0.05**

Figure 1 Measurement of strawberry spread gel stiffness.

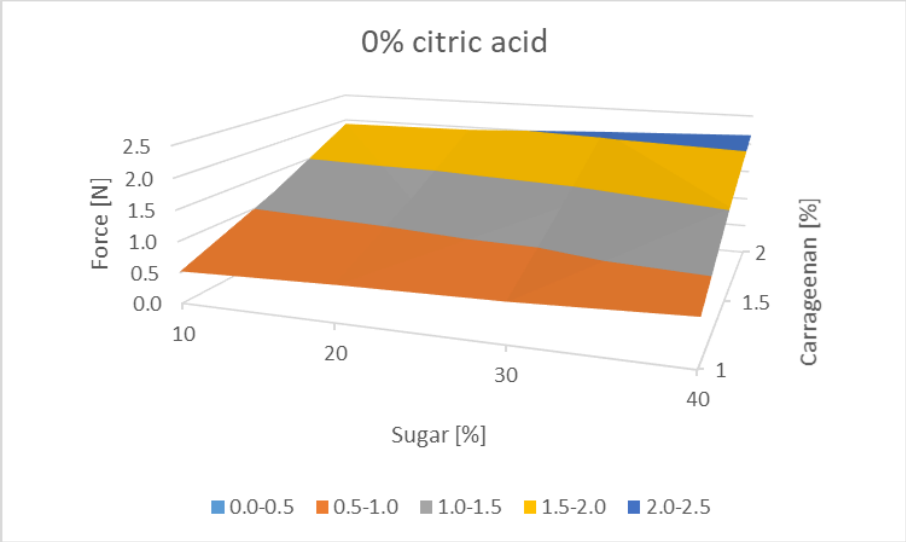

Figure 2 Dependence of gel stiffness on sugar and carrageenan C content, content of citric acid = 0.00%.

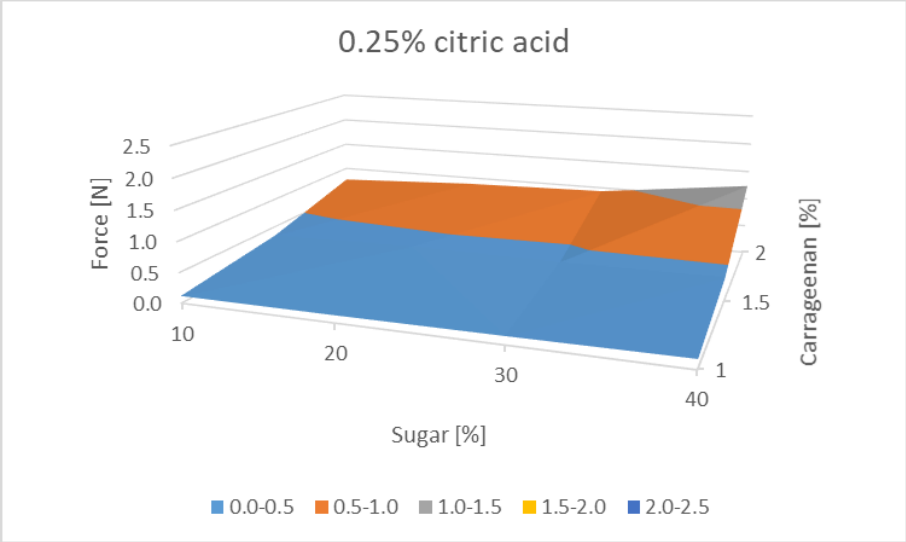

Figure 3 Dependence of gel stiffness on sugar and carrageenan C content, content of citric acid = 0.25%.

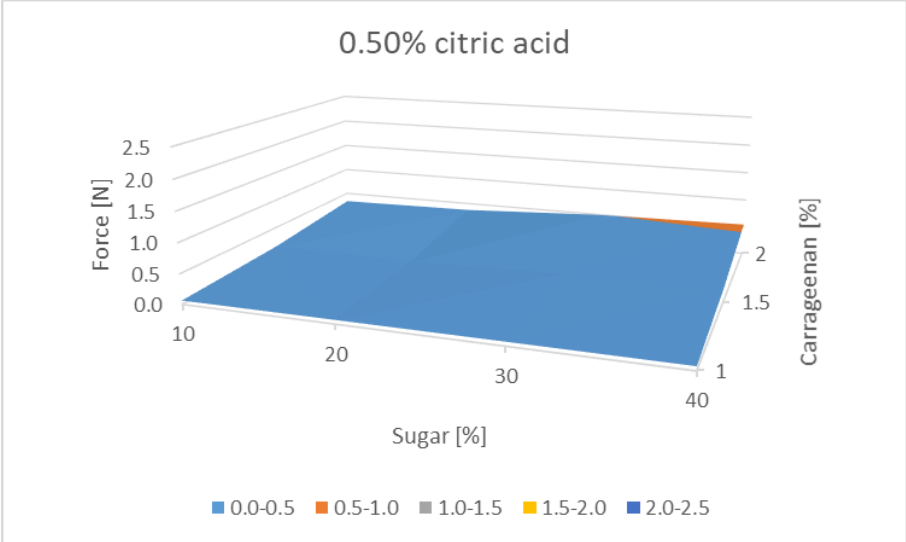

Figure 4 Dependence of gel stiffness on sugar and carrageenan C content, content of citric acid = 0.50%.

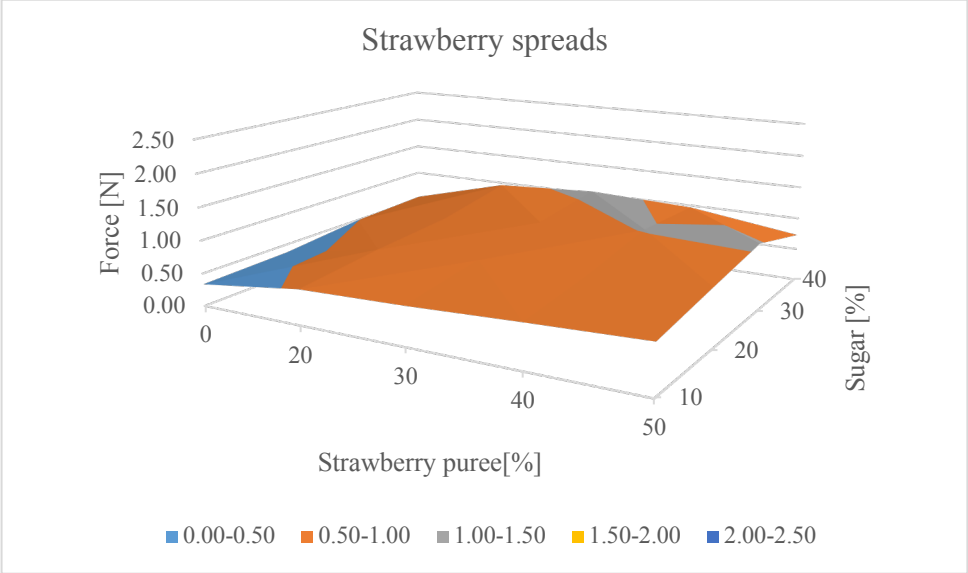

Figure 5 Dependence of strawberry spread stiffness on the content of strawberry puree and sugar, addition of carrageenan C = 2% and addition of citric acid to the total content of 0.50%.

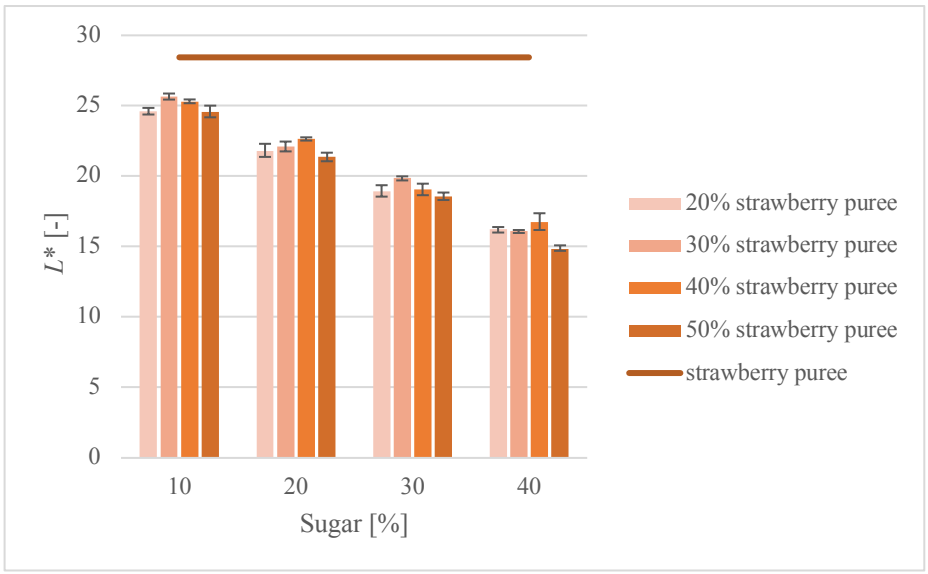

Figure 6 Dependence of L* on sugar content and strawberry puree content in the samples J1 – J16 in comparison with strawberry puree.

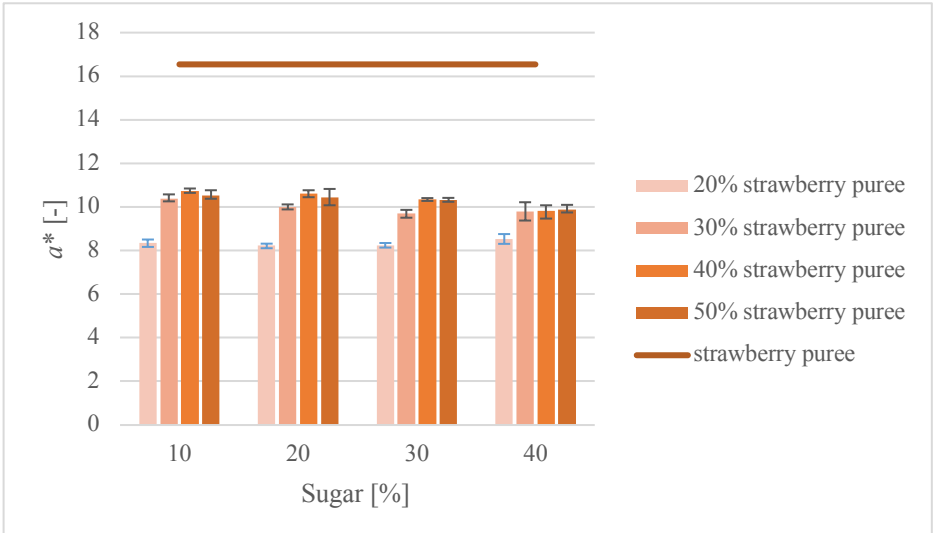

Figure 7 Dependence of a* on sugar content and strawberry puree content in the samples J1 – J16 in comparison with strawberry puree.

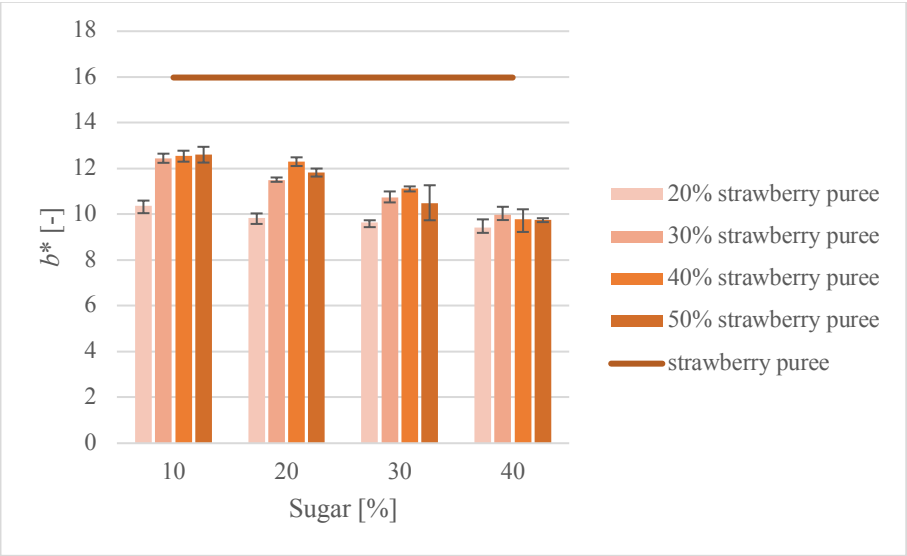

Figure 8 Dependence of b* on sugar content and strawberry puree content in the samples J1–J16 in comparison with strawberry puree.

Table 3 Sensory analysis results of the strawberry spread samples (J1 – J16).

Sample	Sensory attributes				
	Texture	Sweet taste	Sour taste	Fruity taste	Overall acceptability
J1	4.00 ±1.56[b]	3.70 ±1.77[c]	3.20 ±1.99	2.30 ±1.70[a]	3.35 ±2.86
J2	4.60 ±1.58[a]	5.80 ±1.55[b]	3.60 ±2.12	3.80 ±2.15	4.87 ±2.65
J3	5.40 ±1.96	7.00 ±1.94[c]	3.70 ±1.34	4.00 ±1.56[a]	4.92 ±2.56
J4	5.50 ±1.27[a]	7.70 ±1.49[c]	3.70 ±1.34	4.00 ±1.49[a]	4.20 ±2.83
J5	4.50 ±1.51[a]	4.00 ±1.70[c]	3.20 ±2.15	2.80 ±1.40	4.00 ±3.03
J6	4.90 ±1.60	6.10 ±1.79[b]	3.60 ±1.43	3.60 ±1.26	4.95 ±2.60
J7	5.80 ±2.20[a]	6.90 ±1.85[c]	3.00 ±1.56	3.90 ±1.60[a]	4.25 ±2.41
J8	6.20 ±1.48[b]	7.60 ±1.58[c]	3.40 ±1.26	3.90 ±1.45[a]	4.51 ±2.37
J9	4.30 ±1.77[a]	4.90 ±1.29[c]	3.30 ±1.83	3.20 ±1.23	4.29 ±2.88
J10	5.00 ±1.76	6.00 ±1.49[b]	2.50 ±1.35	3.70 ±1.34	4.60 ±2.41
J11	6.10 ±2.18[a]	6.80 ±2.20[b]	2.60 ±1.51	3.40 ±1.43	3.82 ±2.62
J12	6.00 ±1.76[a]	7.50 ±1.96[c]	2.50 ±1.51	3.60 ±1.78	4.05 ±2.82
J13	4.70 ±1.83	4.50 ±1.65[c]	2.50 ±1.65	3.00 ±1.83	4.14 ±2.95
J14	5.00 ±1.70	5.90 ±2.38[a]	2.70 ±1.89	3.30 ±2.26	4.48 ±2.96
J15	6.20 ±2.39[a]	7.50 ±1.78[c]	2.70 ±1.77	3.30 ±1.77	3.53 ±2.54
J16	5.70 ±1.70[a]	8.30 ±1.83[c]	2.70 ±1.49	3.60 ±1.78	3.91 ±3.16

Note: [a] Significant difference by Student's t-test (p = 0.05), [b] Significant difference by Student's t-test (p = 0.01), [c] Significant difference by Student's t-test (p = 0.001).

The results show that it is not necessary to add citric acid to strawberry spreads because of the natural occurrence of organic acids in a strawberry puree to improve the texture parameters **(Kallio et al., 2000; Sturm, Koron and Stampar, 2003)**. However, the addition of citric acid is necessary for the sensory parameter of the final product (balance of sweet and sour taste). Therefore, the same final concentration of 0.5% of citric acid was chosen for the preparation of all strawberry spreads.

One of the parameters of the overall sensory perception of this type of product for the consumer is gel stiffness **(Pérez-Herrera et al., 2020)**. The results of the strawberry spreads gel stiffness (Figure 5) show, that both, sugar and strawberry puree content contribute to this ability to form a gel (p = 0.05). The maximum force required to compress

the gel was higher than that of carrageenan gels with the same sugar and citric acid addition (p = 0.05). Samples containing 40 – 50% of strawberry puree and 30 – 40% of sugar achieved the highest gel stiffness. Only samples without strawberry puree and low sugar content (10 – 20%) showed low gel stiffness.

Colour is one of the most important parameters of strawberries and strawberry products for their final perception and attractiveness to consumers **(Bursać Kovačević et al., 2015)**. Parameter (L^*) of prepared strawberry spreads J1 – J16 is shown in Figure 6. The results show that the addition of sugar significantly reduces product lightness (p = 0.001). This fact is probably due to the amount of sugar, which contributes to the Maillard reaction **(Li et al., 2020; Liao et al., 2020; Shen, Chen and Li, 2018; Basu, Shivhare and Singh, 2013)**. The addition of strawberry puree also had a significant effect on parameter L^* of strawberry spreads (p = 0.05), but less than that of sugar.

The parameter a^* (redness, Figure 7) was not affected by the amount of sugar added (p = 0.05). Significantly lower was the redness (a^*) of the strawberry spreads with a strawberry puree content of 20%. On the other hand, the addition of strawberry puree affected the colour parameter a^* (p = 0.05).

Colour parameter b^* (yellowness, Figure 8) was lower for samples with a strawberry puree content of 20% (p = 0.05). Like the parameter L^*, parameter b^* decreased with the addition of sugar. This difference is statistically significant (p = 0.05).

The results of the sensory analysis of strawberry spreads (J1 – J16) are shown in Table 3. It is visible, that the evaluation of all samples was very subjective. The results of sensory evaluation of texture and sweet taste show that panellists identify differences between samples. In contrast to this fact, the differences in acid taste and overall acceptability (p = 0.05) were not perceived between samples. The overall acceptability values ranged across the whole hedonic scale for all samples. No statistically significant differences between the overall acceptability value of tested samples were observed (p = 0.05).

CONCLUSION

The reduction of sugar content in strawberry spreads was carried out with a focus on people with special nutritional requirements, specifically suffering from obesity and type 2 diabetes. Carrageenan is a suitable gelling agent for the preparation of reformulated strawberry spreads. The applicable concentration of carrageenan C in strawberry spreads for the ideal stiffness of strawberry spreads was 2%. The results of the maximum compression force show a statistically significant increase of gel stiffness (p = 0.05) with the increasing addition of strawberry puree. As the sugar content in the model increases, the maximum compression force values increase. This effect is also statistically significant (p = 0.05) but not as much as the effect of strawberry puree. It was possible to reduce the sugar content in strawberry spreads up to 10% with acceptable sensory perception. The production of this type of fruit spreads with standard characteristics is a complex task influenced by many factors, including the type of used gelling agent and characteristics of input raw material. These reformulated strawberry spreads may be an alternative to jams, which are not recommended because of their high sugar content, for people suffering from these diseases.

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EFFECTS OF THE LACTATION PERIOD, BREED AND FEED ON AMINO ACIDS PROFILE OF MARE'S MILK

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ABSTRACT

The effects of the lactation period, breed, and feed on amino acids profile of mare's milk were investigated. The feed contained two major essential amino acids (EAAs) leucine (7.31 – 10.3 g.kg^{-1}) and arginine (6.37 – 9.59 g.kg^{-1}); it also included minor EAAs methionine (2.11 – 3.05 g.kg^{-1}) and histidine (2.48 – 3.60 g.kg^{-1}). Glu+Gln, Asp+Asn, and proline, major nonessential amino acids (NEAAs), constituted approximately 60% of total NEAAs (TNEAAs). The ratio of total EAAs to NEAAs ranged from 1:1.2 to 1:1.4. Amino acids (AA) content throughout all milk samples varied due to mare's different conditions and lactation days. Except for the 1P milk sample, total AA content in the 2 – 8Ps specimens caused by differences in breed oscillated from the 2nd to 28th day of lactation within the following limits: 21.9 – 54.6 g.kg^{-1}, 33.6 – 70.7 g.kg^{-1}, 38.1 – 71.2 g.kg^{-1}, 29.46 – 74.2 g.kg^{-1}, 52.2 – 87.1 g.kg^{-1}, 37.9 – 70.3 g.kg^{-1} and 26.4 – 64.5 g.kg^{-1}, respectively. In relation to TEAAs in milk, the highest EAAs levels were reached in arginine, leucine and lysine ranging between 2.41 – 4.35 g.kg^{-1}, 3.36 – 5.59 g.kg^{-1} and 2.72 – 4.80 g.kg^{-1}, respectively, while the lowest AAs amounts were indicated in histidine and methionine, 0.91 – 1.58 g.kg^{-1} and 1.23 – 2.04 g.kg^{-1} respectively. Total NEAAs content was slightly higher than that of EAAs; the TNEAAs to TEAAs ratio was 1:0.9 proximately. Glu+Gln, Asp+Asn and proline were determined as major NEAAs of milk ranging between, 6.77 – 11.0 g.kg^{-1}, 3.21 – 5.60 g.kg^{-1} and 1.25 – 2.18 g.kg^{-1}, respectively; levels of NEAAs such as cysteine and glycine oscillated between 0.89 – 1.52 g.kg^{-1} and 0.64 – 1.15 g.kg^{-1}, respectively. The average TAAs contents caused by breed differences were 62.8 g.kg^{-1}, 42.8 g.kg^{-1}, 44.7 g.kg^{-1} and 44.8 g.kg^{-1}, respectively, on the 2nd, 5th, 10th, and 28th lactation days.

Keywords: mare's milk; amino acids; minerals; lactation; feed; breed

INTRODUCTION

Milk is a fluid secreted by the female of all species of mammals. There are about 4500 species producing milk to cover the complete nutritional requirements of the neonate of the species, as well as to provide some immunological protection and to satisfy other physiological requirements. The milk of all species is similar but there are significant species-specific differences. Interspecies differences in the quantitative composition of milk probably reflect differences in the metabolic processes of the lactating mother and the nutritive requirements (amino acids, minerals, fatty acid, vitamins, etc.) of the sucking young. In addition to supplying all the nutritional requirements of the neonate, many of the micro-constituents of milk, such as oligosaccharides, immunoglobulins, metal-binding proteins, and enzymes serve protective roles. Because the nutritional and physiological requirements of each species are rather unique, the composition of milk shows substantial inter-species differences **(Fox, 2009; Fox and Mcsweeney, 1998; Wong et al., 1999)**.

Milk of only about 180 species of mammals has been analyzed up to now; the data for only about 50 species are considered reliable **(Fox and Mcsweeney, 1998; Park, 2009)**. Besides being the most important nutritional resource for foals during the first months of life, the mare´s milk is also one of the most important basic foodstuffs for human populations in Kazakhstan, Kirghizia, Tadzhikistan, Uzbekistan and Mongolia. Lactic-alcoholic beverages called Koumiss, Airag and Kumis are traditionally produced through fermentation **(Di Cagno et al., 2004; Montanari et al., 1996; Malacarne et al., 2002; Orskov, 1995; Pikul and Wójtowski, 2008)**. To a lesser extent, horses have been used as dairy herds in Eastern and Central Europe (Belarus, Ukraine, Hungary, and Bulgaria) **(Caroprese et al., 2007; Doreau and Martin-Rosset, 2002; Fox, 2009)**. Regarding Western Europe, where the most important product of equine breeding is represented by the foal, studies on mare's milk have concentrated mainly on the growth and health of the newborn horse, however, in two decades, the mare's milk has been subjected to through research and, as the result, consumed for human health and as a fundamental food **(Park, 2009)**. Besides the composition of mare's milk fat, the properties of its protein fractions and composition of

amino acids suggest that this product is more similar to human milk than to cow's milk. For the above reason, and because of the low cross-reactivity between cow's and mare's milk proteins, a clinical study has suggested that mare's milk could be used as a valid replacement for cow's milk in children with severe IgE-mediated cow's milk protein allergy **(Businco et al., 2000)**. More recently, the consumption of milk and other dairy foods, by virtue of their mineral, bioactive lipids, and protein components, has been shown to help reduce the risk of chronic disease disorders including osteoporosis, hypertension, excess body weight and body fat, dental caries, and some types of cancer **(Csapó et al., 1995; Curadi et al., 2001; Miclo et al., 2007)**.

The nutritional role of mare's milk as a component of the diet has traditionally been evaluated based on its overall contribution of essential and non-essential nutrients forming a high-quality diet for the support of optimal growth and development of the foal. The colostral period of mares is much shorter than that of cows and the colostrum shows significant differences from common milk only on the first day after foaling **(Barello et al., 2008; Huth, DiRienzo and Miller, 2006)**. The nutritional dependence of the foal on mare's milk composition seems to be most marked immediately after birth. In the case of dairy animals, it is well known that nutrients digested by the dairy cows have a significant effect on the maternal milk composition **(Huth, DiRienzo and Miller, 2006)**. For the proper nutritional management of horses (during the lactating period and of foals during the sucking period), it is essential to clarify the relationship between the intake of nutrients and the milk composition in mares. The daily nutrient requirements of lactating mares are very high, and they can be compared with those of racing horses in heavy training. Important nutrients are secreted by the mare to supply her foal with energy, proteins, fats, carbohydrates, vitamins, and minerals for optimal development and growth. To substitute these nutrient losses and at the same time to support maintenance requirements, lactating mares must consume adequate amounts of quality feeds **(Pagan and Hintz, 1986)**. It is known that the intake of minerals and amino acids is particularly important for the growth of foals. The protein requirement of the horse is influenced by the capacity of the protein to satisfy the amino acid needs of the animal. In the horse diet, there are currently four main essential amino acids: lysine, methionine, threonine, and tryptophan. These amino acids are the most important nutrients because limiting their levels will affect the growth and development of the horse.

In the last decade, quite detailed research on equine milk and colostrum composition and, especially studies on proteins, lipids, and minerals have been carried out for the above-mentioned reasons. Many authors have reported data on the profile of essential nutrients such as minerals, amino acids, and vitamins in mare's milk; however, no complex research including all the above factors has been implemented **(Fišera et al., 2018)**. Moreover, there are a few studies of the relationship between changes in mare's milk main components and the above affecting factors such as lactation stage and others.

Scientific hypothesis

Therefore, this study is focused on changes in contents of essential nutrients detected in mare's milk depending on lactation stages, feeding system, and on some breeds. The research aimed to analyze the content of significant nutritional components in mare's milk and lactating mare´s feed as well as to characterize the effects of several factors that affect milk properties such as lactation periods, breed, and mare's diet. More specifically, the aims of the study were i) to analyze changes in the major amino acid composition of mare's milk caused by differences in broodmares and by periods in the first month after parturition, ii) to analyze the major amino acid content and dry matter levels in the lactating broodmares´ feed during the lactation days after parturition and iii) to study the relations between the amino acid composition of milk and feed. The final goal was to get detailed information on the relationship between amino acids detected in mare's milk and their feed. Effects of the lactation period, breed, and feed on amino acids profile of mare's milk were investigated and successively evaluated.

MATERIAL AND METHODOLOGY

Selection of broodmares and broodmares' milk samples

To collect mare's milk and colostrum samples, eight different mature, well-developed, and normal broodmares were selected. All the selected mares were from seven to sixteen years of age (10 ±3 parities), with the live weight between 500 and 600 kg. They were kept indoor and outdoor individually and fed with 1.3 kg wheat bran, 2.1 kg hominly, 5.0 kg oats every day. Water was available from troughs at all times. All mares were vaccinated against influenza and herpes virus per 6 months.

The milk samples (approximately 40 – 50 mL) from selected broodmares were taken into plastic containers on 2, 5, 10, 28, and 56 days postpartum; 31 individual samples were collected in total (Table 1). Before milking, foals were separated from their mothers for approximately 2 hours to prevent suckling. The first milking of mares was carried out with hands as deep as possible. Some mares had not previously been subjected to any milking procedures. The second milking was done accordingly. Collected milk samples were taken directly to the laboratory and then immediately stored and frozen at -20 °C and -25 °C. The samples of feed mixture for the lactating mares (in the amount of approximately 500 g) were collected into polyethylene bags on the days of milk sample collecting altogether 29 samples were collected (Table 1). Then all the samples were taken to the laboratory and stored at room temperature.

All the collected milk samples were being frozen in a freezer at -80 °C for 4 hours, and then they were being lyophilized at 120 Pa and -50 °C for 2 days using vacuum lyophilization (Labicom, Czech Republic). All the lyophilized samples were stored in a fridge at -4 °C before their analysis. All the feed samples were being mixed or ground using a mixer for 3 min and then put into plastic containers. All the grounded samples were stored at room temperature prbeforeheir analysis.

Reagents and solutions: All reagents used were of analytical reagent grade (Sigma-Aldrich, St. Lois, Mo, USA). Concentrated hydrochloric acid 37% (v/v) HCl, 6 mol.L^{-1} HCl, argon gas, 0.1 mol.L^{-1} HCl, loading buffer of pH 2.2, 30% (v/v) H_2O_2, 85% (v/v) HCOOH.

Equipment: The contents of amino acids in all samples were determined by using an AAA 400 Amino Acid Analyzer (INGOS, Prague, Czech Republic), a liquid chromatograph designed for the analysis of amino acids on an ion-exchanger column with post-column derivatization by means of ninhydrin and for the determination of biogenic amines. A thermoblock (Labicom, Olomouc, Czech Republic), a rotary evaporator (Heidolph Instruments GmbH + Co. KG, Kelheim, Germany) and an oil bath (Memmert GmbH + Co. KG, Schwabach, Germany) were used for acid and alkaline oxidizing hydrolyses of all milk and feed samples.

Procedures

Analysis of dry matter

Dry matter (DM) was determined according to the AOAC standard procedure **(AOAC, 1990)**. Contents of dry matter were calculated according to the following equation DM (%) = $m_2.100/m_1$, where DM (%) is the percentage of a total dry matter, m_1 is sample weight before DM determination and m_2 is sample weight after drying.

Analysis of amino acid contents

The contents of amino acids in all samples were determined by using an AAA 400 amino acid analyzer (INGOS, Prague, Czech Republic). An amino acid analyzer is a special compact liquid chromatograph designed for the analysis of amino acids on an ion-exchanger column with post-column derivatization by means of ninhydrin and for the determination of biogenic amines.

The acidic hydrolyses of all milk and feed samples were performed with few modifications under hydrolysis time and temperature conditions according to **Buňka et al. (2009)** and **Buňka, Hrabě and Kráčmar (2004)**. Briefly, the lyophilized milk (50 – 60 mg) and dried feed samples (80 – 90 mg) were accurately weighed in screw-capped test tubes for acidic hydrolysis (capacity of 20 mL) with Teflon caps. 15 mL HCl (6 mol.L^{-1}) solutions were added to every tube with a sample. Afterward, the tubes were being purged by blowing with argon gas for approximately 20 seconds, and then the vacuum-sealed tubes were being heated in a thermoblock at 117 ±1 °C for 23 hours. The temperature of the thermoblock was independently controlled by using a thermometer immersed in a test tube filled with silicone oil (the test tube was placed in the thermoblock). After hydrolysis, hydrolyzed samples were filtered using a paper filter and washed with 0.1M HCl. Then the filtrates were firstly dried using a rotary evaporator at 50 °C and 90 rpm and evaporated secondly and thirdly after a double washing with distilled water (approximately 25 mL) until we obtained a dried mass. The dried mass was dissolved in loading buffer with pH 2.2 and made up to the mark in a 25 mL volumetric flask. Then 1.5 mL of this solution was put into a plastic container and filtered through a 0.45 μm filter and subsequently placed in a fridge until loaded to the amino acid analyzer. All analyses were performed in triplicate for every sample.

In order to determine sulphur-containing amino acids, all samples were hydrolyzed separately with HCl (6 mol.L^{-1}) after oxidizing the samples with performic acid according to methods elaborated by **Buňka, Hrabě and Kráčmar (2004)** and **Amarakoon (2009)** with few modifications. Performic acid was prepared in the ratio of 1:9 from 30% H_2O_2 and 85% HCOOH. After incubation at room temperature for 2 hours, the performic acid was being kept in a fridge at 4 ±1 °C for approximately 30 seconds. The lyophilized milk (50 – 80 mg) and dried feed samples 1000 – 8000 mg) were accurately weighed and put into a 250 mL Erlenmeyer flask. 15 mL of performic acid was added to each sample and then it was being cooled at 4 ±1 °C at least for 16 hours for sample oxidizing. After oxidation, 1 – 2 mL of concentrated HCl was added to the oxidized samples and incubated in the hood for approximately 30 min until chlorine gas was removed. Then 150 mL of 6 mol.L^{-1} HCl was added to the oxidized samples and heated in an oil bath at 117 ±1 °C for 23 hours. After hydrolysis, the samples were filtered with a paper filter, dissolved in 0.1 mol.L^{-1} HCl and made up to the 250 mL mark in a volumetric flask. 25 mL of the diluted sample was transferred with a 25 mL pipette into a flask and then the hydrochloric acid was evaporated in the rotary evaporator after washing it twice with distilled water until a dried mass was obtained. Then the dried mass was dissolved in loading buffer with pH-2.2 and made up to the mark in a 25 mL volumetric flask. Then 1.5 mL of this solution was put into a plastic container after filtering through a 0.45 μm filter and then placed in a fridge before loading it to the amino acid analyzer. All analyses were performed in triplicate for every sample.

Determination of amino acids

A standard mixture of 17 analyzed amino acids (aspartic acid + asparagine, threonine, serine, glutamic acid + glutamine, proline, glycine, alanine, valine, isoleucine, leucine, phenylalanine, tyrosine, histidine, lysine, arginine, methionine, and cystine) was obtained from INGOS (Prague, Czech Republic). The content of amino acids was determined by an AAA 400 device according to the manufacturer´s program. Then the column was being regenerated with 0.2 mol.L^{-1} NaOH for 10 min and stabilized for further 17 min with buffer A. The temperature of the column was set to 60 °C for a specific time slot (0 – 60 min and 90 – 102 min) and to 74 °C in the mean time (60 – 90 min). The following flow rates were employed: 0.3 mL.min^{-1} for buffers and 0.2 mL.min^{-1} for ninhydrin reagent. Each hydrolysate was analyzed in duplicate.

Statistical analysis

All the contents of total amino acids in all the analyzed samples were expressed as mean ±SD and calculated based on retention time obtained in chromatograms **(Buňka, Hrabě and Kráčmar, 2004; Ingos Ltd., 2006)**. The data were evaluated by summary statistics and the variance was calculated using an ANOVA procedure performed using Statistica® 12.0 software (StatSoft, USA). All data obtained by the AAA 400 were expressed as the mean levels in fresh milk using the following equation

TA = $A_0.\varepsilon_0$ = (L-C)/(D-C), where TA is amino acid content in fresh milk, A_0 is amino acid content of lyophilized milk, ε_0 is correlation coefficient, L is fresh milk weight, D is lyophilized milk weight and C is container weight.

RESULTS AND DISCUSSION

Dry matter in the feed

The dry matter (DM) content in all feed samples for the lactating mares is shown in Table 2. The percentage of DM content varied slightly in individual feed samples due to preparation conditions of feed mixture and because of the ratio of forages in the feed mixture. The average DM content oscillated between 88.6% and 90.6%. According to some researchers **(Looper et al., 2001),** the DM content in selected high-quality feed types for the lactating mares such as grains, hay, and chaff ranged from 87 to 91 % (w/w). The above results comply with NRC **(Pagan and Hintz, 1986)**. DM content in all the feeds, therefore, is in accordance with the standard level of DM in feed and forage for the lactating mare.

Amino acid composition of feed and milk

The amino acid content in the feed

In the experiment, the essential amino acids and non-essential amino acid profiles of each investigated feed for the lactating mare were determined. All the data are expressed as mean ±SD in g.kg^{-1} (p <0.005) and they also depict considerable variations in the amino acid content in feeds. The protein nutritive value is given by its amino acid profile. The protein contents in horse feeds such as in grains, hay, chaff, pasture, or in others range from 10 to 25% of total weight depending on the type of feeds **(Pagan and Hintz, 1986)**. Out of 20 amino acids, a human being or an animal can synthesize only nine. The remaining amino acids should be provided in their food. Arginine is regarded as a semi-essential amino acid **(Boisen, Hvelplund and Weisbjerg, 2000)**.

As shown in tables, AA contents in feeds for the lactating mares differed, which could have been caused by some of the above mentioned factors. Generally, NEAA glutamic acid (Glu+Gln) content (18 – 21 g.kg^{-1}) reached the highest values while the EAA methionine and histidine levels
(2.5 – 3.6 g.kg^{-1}) dropped the lowest among all feeds. EAA arginine and leucine, NEAA aspartic acid (Asp+Asn) and proline content in all feeds were higher than levels of other AAs, 7 – 9 g.kg^{-1}, 8 – 10 g.kg^{-1}, 7 – 9 g.kg^{-1} and 8 – 9 g.kg^{-1} respectively. In the animal diet, there are currently recognized four main essential amino acids: lysine, methionine, threonine, and tryptophan, which are the most important nutrients; limiting their levels will affect the growth and development of the horse **(Saastamoinen and Koskinen, 1993)**. Particularly in the horse, the daily lysine requirement for the lactating mare ranges between 13.8 – 16.9 g per 100 kg of body weight **(National Research Council. 2007)**. The contents of these above AAs in feeds were, except for tryptophan, which was not analyzed, relatively lower than the amounts of the others. Lysine levels ranged between 3.0 – 4.5 g.kg^{-1}, methionine amounts oscillated between 2.5 – 3.6 g.kg^{-1} and threonine content ranged between 3.4 – 4.0 g.kg^{-1}.

Regarding other AA such as EAA isoleucine, valine and phenylalanine, NEAA cysteine, glycine, serine and tyrosine, their levels did not differ much ranging between 3.4 g.kg^{-1}, 4.8 – 6.2 g.kg^{-1}, 4.7 – 6.0 g.kg^{-1}, 3.4 – 3.8 g.kg^{-1}, 5 – 6 g.kg^{-1}, 4.5 – 5.2 g.kg^{-1} and 3.4 – 7.0 g.kg^{-1}, respectively. According to some reports, tyrosine and cysteine are regarded as semi-essential amino acids, since they can be synthesized exclusively from methionine and phenylalanine, respectively **(Boisen, Hvelplund and Weisbjerg, 2000)**. Total NEAA amounts (approximately 50 – 66 g.kg^{-1}) in all feeds on all days were slightly higher than total EAA levels (approximately 40 – 51 g.kg^{-1}). The highest total EAA content reached a maximum (51.7 g.kg^{-1} in 5F) on the 2nd day while the lowest value was found in 1F on the 2nd day (40.5 g.kg^{-1}). In the case of NEAA, the highest amount increased to 66.8 g.kg^{-1} in 7F on the 2nd day and the lowest level was 50.4 g.kg^{-1} in 2F on the 5th day. The above amounts were comparable to levels found in some types of feeds such as oats, barley, sorghum and wheat **(Awadalkareem, Mustafa and El Tinay, 2008; Pagan and Hintz, 1986)**. The protein requirement of the horse is influenced by how well the protein provides for the amino acid needs of the animal; some EAAs are limiting essential amino acids in grass-based feeding programs. It has been reported that daily protein intake of the lactating mare averages at 1493 g.day^{-1} **(Pagan and Hintz, 1986)**. Levels of TAAs in examined feeds ranged between 92 – 116 g.kg^{-1}; their amount was higher than that in some species of oats and there was an agreement with their amount contained in good quality feed for animals **(Biel, Bobko and Maciorowski, 2009)**.

Average amino acid composition of feed

The average content of all AAs examined in 1F – 8F on different lactation days is summarized in Table 3. The average content of each amino acid was calculated in relation to amounts of the AA revealed in all feed on the 2nd, 5th 10th and 28th, days respectively. As shown in this table, all AA contents, except for EAA leucine and methionine and for NEAA proline, on the 28th day and in feeds on the 5th and 28th days slightly decreased. It was observed that the AA contents in feeds reached the highest levels on the 2nd day while the lowest amounts were detected in feeds on the 5th day. Nevertheless, EAA phenylalanine content on the 10th day was higher than the amount of the others. The contents of arginine and leucine, the major EAAs in feed, grew highest ranging between 7.70 – 8.42 g.kg^{-1} and 8.66 – 9.35 g.kg^{-1}, respectively.

Other EAA contents were two and three times lower when compared to major EAA amounts. Moreover, Glu+Gln, proline, alanine and Asp+Asn dominated among the NEAA in feeds ranging between 18.5 – 19.9 g.kg^{-1}, 7.31 – 8.30 g.kg^{-1}, 6.22 – 6.52 g.kg^{-1} and 7.90 – 8.36 g.kg^{-1}, respectively. Contents of the other AA did not differ mutually; methionine and histidine levels in feed scored lowest. Therefore, the total EAA content in all feeds was 1.2 times lower than NEAA content; the average ratio of EAA to NEAA was 1:1.2 (44.1 – 47.0 g.kg^{-1}: 56.4 – 61.2 g.kg^{-1}). It was proved that all feeds for the lactating broodmares were rich in EAA.

Table 1 Milk and feed sample characteristics.

Milk	Days postpartum					Feed	Days postpartum				
	2nd	5th	10th	28th	56th		2nd	5th	10th	28th	56th
1P	+	+	+	+	-	1F	+	+	+	+	+
2P	+	+	+	+	+	2F	+	+	+	+	-
3P	+	+	+	+	-	3F	+	+	+	+	-
4P	+	+	+	+	-	4F	+	+	+	+	-
5P	+	+	+	+	-	5F	+	+	+	-	-
6P	+	+	+	-	-	6F	+	+	+	-	-
7P	+	+	+	-	-	7F	+	+	+	-	-
8P	+	+	+	+	-	8F	+	+	+	-	-

Note: + sample was taken; - sample was not taken.

Table 2 The dry matter content of feed (%, w/w).

Feed	Dry matter (mean value ±SD)				
	2nd	5th	10th	28th	56th
1F	89.38 ±0.19	89.32 ±0.15	89.04 ±0.51	88.96 ±0.66	89.49 ±0.51
2F	90.56 ±0.67	88.64 ±0.34	89.54 ±0.37	89.36 ±0.55	n.a.
3F	90.32 ±0.54	89.55 ±0.86	88.69 ±0.98	89.04 ±0.22	n.a.
4F	88.89 ±0.42	89.14 ±0.51	89.06 ±0.20	88.90 ±0.08	n.a.
5F	89.64 ±0.40	88.99 ±0.70	88.65 ±0.68	n.a.	n.a.
6F	90.12 ±0.39	88.80 ±0.57	88.81 ±0.58	n.a.	n.a.
7F	90.47 ±0.31	89.37 ±0.37	88.81 ±0.53	n.a.	n.a.
8F	90.00 ±0.63	88.80 ±0.39	88.7 ± .51	n.a.	n.a.

Note: F – feed samples, SD – standard deviation, n.a. – not analyzed.

Table 3 The average content of each amino acid in 1F – 8F samples feed.

Amino acid (g.kg^{-1})	1F – 8F							
	2nd day		5th day		10th day		28th day	
	mean	SD	mean	SD	mean	SD	mean	SD
Arginine	8.42	0.82	7.91	0.89	8.18	0.84	7.70	0.74
Histidine	3.15	0.32	2.83	0.29	2.95	0.30	2.88	0.29
Isoleucine	4.09	0.36	3.91	0.38	4.04	0.40	3.91	0.27
Leucine	9.35	0.77	8.66	0.80	8.98	0.90	8.99	0.71
Lysine	4.31	0.42	4.00	0.50	4.18	0.47	3.77	0.35
Methionine	2.49	0.26	2.32	0.09	2.38	0.09	2.38	0.21
Phenylalanine	5.45	0.51	5.32	0.60	5.49	0.62	5.32	0.47
Threonine	3.89	0.32	3.63	0.41	3.77	0.38	3.69	0.35
Valine	5.87	0.55	5.55	0.56	5.73	0.61	5.57	0.46
TEAA	**47.02**	**4.34**	**44.13**	**4.51**	**45.70**	**4.61**	**44.21**	**3.85**
Alanine	6.52	0.52	5.99	0.60	6.22	0.59	6.16	0.56
Asp+Asn	8.36	0.71	7.90	0.81	8.13	0.81	7.91	0.61
Cysteine	3.57	0.13	3.52	0.13	3.52	0.22	3.46	0.18
Glu+Gln	19.94	1.45	18.45	1.66	19.23	1.69	18.75	1.54
Glycine	5.69	0.50	5.26	0.57	5.48	0.53	5.29	0.52
Proline	8.30	0.56	7.31	0.44	7.67	0.33	7.79	0.53
Serine	4.89	0.36	4.55	0.48	4.72	0.45	4.61	0.42
Tyrosine	3.96	1.31	3.37	0.43	3.47	0.40	3.40	0.44
TNEAA	**61.24**	**5.54**	**56.35**	**5.12**	**58.45**	**5.03**	**57.36**	**4.79**
TAA	**108.26**	**9.88**	**100.48**	**9.63**	**104.16**	**9.65**	**101.57**	**8.63**

Note: TEAA – total essential amino acid content, TNEAA – total non-essential amino acid content, TAA – total amino acid content.

The ratio of AA in some feeds for animals such as in oats grain was reported as 1:2.8 (27 g. (16 g N)$^{-1}$: 75 g.(16 g N)$^{-1}$) **(Biel, Bobko and Maciorowski, 2009)**. TAA levels in feeds ranged from 100.48 g.kg^{-1} to 108.26 g.kg^{-1} depending on feeding days. Overall, these AA concentrations in all feeds were considerably higher than those in daily feed intake of lactating mares **(Pagan and Hintz, 1986)**.

The amino acid content in milk

The variations in the amino acid contents in milk samples from the eight lactating mares on several days after parturition are shown in Table 4, as a representative example. As shown in this table, the AA contents in 1P on all days were three times lower than those in other mares while there were significant differences in AA contents in all milk. It was, perhaps, due to sample processing and the mare's physiological conditions. It was observed that the highest AA contents were reached in all milk on the 2nd day of lactation; afterwards, all AA contents dropped considerably on the 5th day. It corresponded to the end of the colostral period.

In many papers, the lactation period is the most significant factor influencing the composition of all mammalian milk, in particular of horse milk; protein content in colostral period is five times higher than that in milk period, due to providing the foal with all nutrients and immune substances during the first few days **(Kráčmar et al., 2005)**. The colostral period of mares was found to be significantly shorter than that of cows; quality of colostrum showed significant differences from that of normal milk only on the first 2 days after foaling **(Salamon et al., 2009)**. The AA contents in 2P, 3P, 4P, and 8P on the 10th day of lactation was increased significantly and then decreased gradually. In regard to 1P, 5P, 6P and 7P, the AA content was continuously decreasing till the 10th day of lactation, although on the 28th day it increased slightly again. These changes agreed with reports on mare's milk composition during the lactation period.

Some authors reported that after parturition, the AA content in mare's milk was decreasing continuously **(Csapó et al., 2009)**. Leucine, lysine, arginine and valine contents in all mares' milk samples were higher than those of other EAAs, ranging within 4.4 – 6.7 g.kg^{-1}, 3.7 – 6.5 g.kg^{-1}, 4.2 – 5.8 g.kg^{-1} and 2.7 – 4.9 g.kg^{-1}, respectively; the leucine content was the highest one while histidine content (1.2 – 2.2 g.kg^{-1}) was the lowest one in all milk samples. In the case of NEAA, Glu+Gln, Proline and Asp+Asn, their contents in milk ranged between 8.8 – 15.8 g.kg^{-1} and 4.7 – 7.6 g.kg^{-1} respectively; they reached the highest levels while glycine and alanine contents oscillating between 0.9 – 1.5 g.kg^{-1} and 1.3 – 1.9 g.kg^{-1}, dropped lowest in all milk samples. The above AA contents profile agreed with that foundby **Matsui, Inoue and Asai (2003)**.

Some authors reported that when the AA composition was expressed as AA g.100g^{-1} protein, the changes were noticeably less apparent; it was due to high amount of free AA in milk during the colostral period; after the colostral period, total free AA concentration decreased to half value from 0.6 g.kg^{-1} to 0.3 g.kg^{-1}. The contents of free AAs in colostrum, except for threonine, serine, and glutamic acid levels, were about twice as high as those in normal milk **(Csapó-Kiss et al., 1995)**. Overall, AA contents in 6P on lactation days were higher than those in the others; oscillations in 3P, 4P, 5P, 7P, and 8P AA during lactation days were comparable to each other and no significant difference was observed. Although the AA amounts in all milk on different lactation days differed from each other, they are suggested to depend on feed and breed differences.

Average amino acid composition of milk

Further, Table 5 shows the average total AA content in milk related to differences between eight broodmares, which were fed with above mentioned good quality feeds during lactation, relative to 2nd, 5th 10th and 28th days of lactation. It means that the content of each amino acid decreased, without any exception, considering the change of colostrum to milk (2nd to 28th day), although, on the 10th day, a slight increase of all AA contents in milk was observed in milk.

Generally, after the 2nd and 5th day of lactation, there was a slight difference in AA content which agrees with the data reported by some authors who suggested no changes in the amino acid composition of mare's milk proteins between the 8th and 45th day of lactation **(Csapó-Kiss et al., 1995; Davis et al., 1994)**. In the diet of the horse and the newborn foal, lysine, methionine, threonine, and tryptophan are considered the most important substances to affect its growth and development **(Saastamoinen and Koskinen, 1993)**. Lysine was detected to be one of the major EAAs in milk while methionine and threonine content were the lowest ones. The content of major EAAs arginine, leucine, lysine, isoleucine and valine on the 2nd day of lactation follow: 4.35 g.kg^{-1}, 5.59 g.kg^{-1}, 4.80 g.kg^{-1}, 3.01 g.kg^{-1} and 3.49 g.kg^{-1}, respectively. On the 28th day of lactation, their levels decreased to 2.41 g.kg^{-1}, 3.36 g.kg^{-1}, 2.72 g.kg^{-1}, 1.79 g.kg^{-1} and 2.10 g.kg^{-1}, respectively.

Regarding the oscillation of NEAA amounts, the pattern similar to that of EAAs was observed; the major NEAA Glu+Gln levels decreased from 11.0 g.kg^{-1} to 6.7 g.kg^{-1} and Asp+Asn content dropped from 5.6 g.kg^{-1} to 3.21 g.kg$^{-1,}$ etc. The decrease of AA content in milk between the 2nd and the 28th day ranged between 30 % and 40 %; it was comparable to a decrease of AA content studied by **Csapó et al. (2009)**. Total EAA content reached 30.3 g.kg^{-1}, 20.5 g.kg^{-1}, 21.2 g.kg^{-1} and 17.7 g.kg^{-1} on the 2nd, 5th, 10^{th,} and 28th days respectively, showing values lower than total levels of NEAA.

Therefore, the average ratio of total EAA to total NEAA content on lactation days was calculated as 1:1.1, which is in disagreement with the 1:2.2 value found by **Csapó et al. (2009)**; moreover, the ratio in colostrum was reported as 1:2 **(Lehtola and Saastamoinen, 2010)**, which was influenced by breeds and their feed. On the other hand, total EAA content was significantly higher, and the total NEAA was lower than the values mentioned in the literature. Total AA contents during lactation days reached 62.8 g.kg^{-1}, 42.8 g.kg^{-1}, 44.7 g.kg^{-1} and 37.1 g.kg^{-1} respectively; they decreased throughout lactation days analogically to those of EAAs and NEAAs.

Table 4 Amino acid content in 1P sample milk.

Amino acid (g.kg^{-1})	1P									
	2nd day		5th day		10th day		28th day		*2 – 28th days*	
	Mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	***mean***	***SD***
Arginine	1.28	0.05	0.82	0.02	0.77	0.04	1.02	0.01	*0.97*	*0.23*
Histidine	0.50	0.01	0.40	0.01	0.29	0.02	0.39	0.01	*0.39*	*0.09*
Isoleucine	0.99	0.02	0.76	0.03	0.58	0.03	0.73	0.04	*0.77*	*0.17*
Leucine	1.92	0.06	1.47	0.03	1.14	0.08	1.47	0.12	*1.50*	*0.32*
Lysine	1.51	0.00	1.18	0.05	0.87	0.05	1.05	0.04	*1.16*	*0.27*
Methionine	0.72	0.02	0.54	0.03	0.41	0.04	0.48	0.02	*0.54*	*0.13*
Phenylalanine	0.89	0.05	0.64	0.07	0.50	0.05	0.70	0.06	*0.68*	*0.16*
Threonine	0.75	0.02	0.66	0.01	0.46	0.03	0.62	0.03	*0.62*	*0.12*
Valine	1.17	0.06	0.93	0.05	0.74	0.09	0.90	0.02	*0.93*	*0.18*
TEAA	**9.74**	**0.28**	**7.40**	**0.32**	**5.76**	**0.43**	**7.37**	**0.35**	***7.57***	***1.67***
Alanine	0.74	0.02	0.52	0.01	0.40	0.02	0.56	0.04	*0.55*	*0.14*
Asp+Asn	1.84	0.06	1.45	0.09	1.00	0.07	1.37	0.05	*1.42*	*0.34*
Cysteine	0.54	0.05	0.41	0.02	0.32	0.02	0.38	0.01	*0.41*	*0.09*
Glu+Gln	3.49	0.09	3.01	0.08	2.28	0.12	3.23	0.29	*3.00*	*0.52*
Glycine	0.40	0.01	0.29	0.03	0.21	0.01	0.29	0.01	*0.30*	*0.08*
Proline	1.60	0.02	1.41	0.10	1.05	0.11	1.43	0.01	*1.37*	*0.23*
Serine	1.06	0.06	0.88	0.02	0.63	0.05	0.88	0.05	*0.86*	*0.18*
Tyrosine	1.01	0.01	0.70	0.02	0.53	0.02	0.74	0.09	*0.75*	*0.20*
TNEAA	**10.69**	**0.32**	**8.68**	**0.38**	**6.42**	**0.41**	**8.88**	**0.56**	***8.67***	***1.78***
TAA	**20.42**	**0.59**	**16.08**	**0.70**	**12.18**	**0.84**	**16.25**	**0.91**	***16.23***	***3.45***

Note: TEAA – Total essential amino acid; TNEAA – Total non-essential amino acid; TAA – Total amino acid. In italic – Average content of each amino acid at total lactation days.

Table 5 Average content of each amino acid in 1P – 8P samples milk.

Amino acid (g.kg^{-1})	1P – 8P							
	2nd day		5th day		10th day		28th day	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Arginine	4.35	1.35	2.72	0.95	2.91	1.21	2.41	1.12
Histidine	1.58	0.51	1.06	0.33	1.08	0.47	0.91	0.44
Isoleucine	3.01	0.97	2.09	0.66	2.17	0.89	1.79	0.83
Leucine	5.59	1.81	3.87	1.19	4.05	1.64	3.36	1.51
Lysine	4.80	1.55	3.20	1.03	3.27	1.34	2.72	1.33
Methionine	2.04	0.64	1.59	0.50	1.43	0.66	1.23	0.55
Phenylalanine	2.82	0.92	1.87	0.63	1.97	0.83	1.63	0.77
Threonine	2.59	0.85	1.75	0.56	1.74	0.69	1.49	0.71
Valine	3.49	1.13	2.37	0.76	2.57	1.06	2.10	0.94
TEAA	**30.28**	**9.75**	**20.51**	**6.60**	**21.19**	**8.78**	**17.65**	**8.18**
Alanine	2.18	0.68	1.40	0.47	1.47	0.59	1.25	0.56
Asp+Asn	5.60	1.75	3.76	1.17	3.87	1.61	3.21	1.46
Cysteine	1.52	0.47	1.18	0.38	1.03	0.41	0.89	0.39
Glu+Gln	11.00	3.65	7.64	2.37	8.18	3.24	6.77	2.93
Glycine	1.15	0.35	0.75	0.24	0.76	0.31	0.64	0.30
Proline	4.99	1.64	3.54	1.03	3.92	1.63	3.17	1.28
Serine	3.30	1.03	2.24	0.69	2.33	0.93	1.95	0.87
Tyrosine	2.76	0.83	1.81	0.58	1.92	0.80	1.58	0.73
TNEAA	**32.51**	**10.39**	**22.33**	**6.93**	**23.47**	**9.53**	**19.46**	**8.50**
TAA	**62.79**	**20.14**	**42.84**	**13.53**	**44.67**	**18.30**	**37.11**	**16.68**

Note: TEAA – Total essential amino acid; TNEAA – Total non-essential amino acid; TAA – Total amino acid.

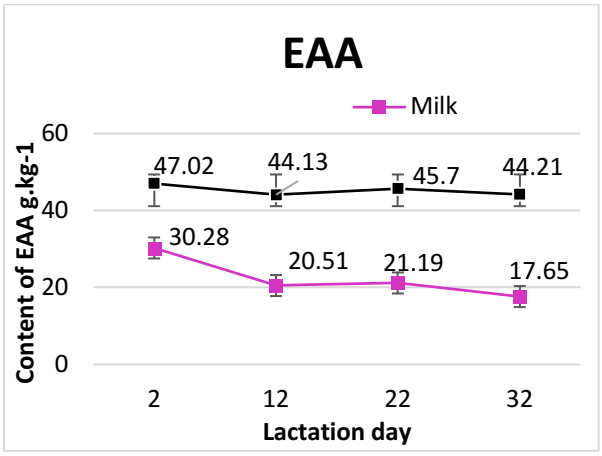

Figure 1 Relation between average total content of essential amino acids in milk and feed.

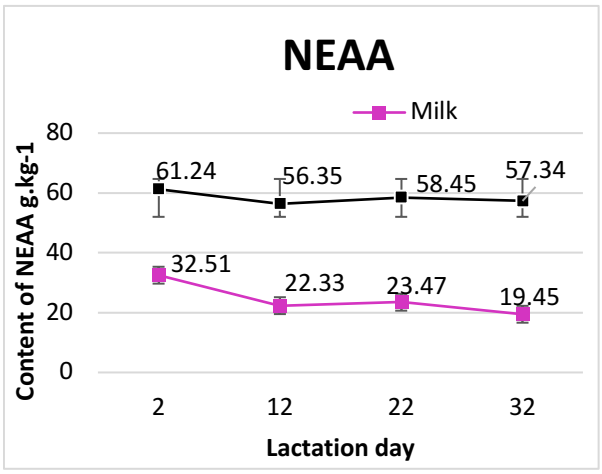

Figure 2 Relation between average total content of non-essential amino acids content in milk and feed.

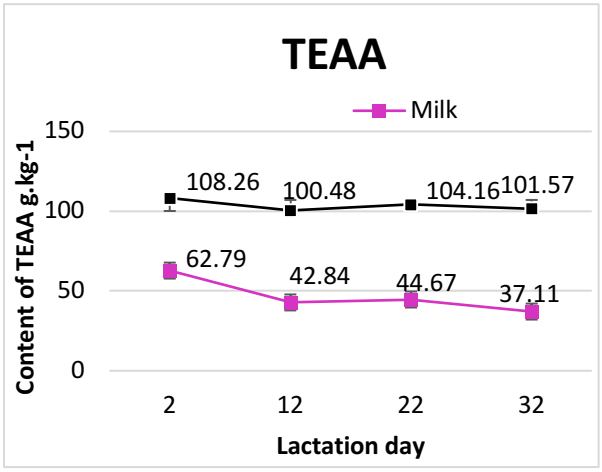

Figure 3 Relation between average total amino acid content in milk and feed.

Some authors reported total AA concentration in mare's colostrum to reach its peak in the first 24 hours after parturition; it ranged from 160 to 170 $g.kg^{-1}$ dropping then to 40 – 42 $g.kg^{-1}$ on the 2 – 5th days, while it decreased to its minimum value of 20 – 25 $g.kg^{-1}$ on 8 – 45th days of lactation **(Csapó-Kiss et al., 1995)**. Equine, milk on the 1st and 2nd day postpartum is considered as initial milk which shows both colostrum and milk properties; therefore, the TAA contents on the 2nd and 5th lactation days did not vary significantly. Nevertheless, TAA levels on the 10th and 28th days were considerably higher than the contents reported above. It has been found by several authors that there were no significant differences in the amino acid composition between the breeds but that total AA content depends on the feeding system and breeds **(Pagan and Hintz, 1986; van den Berg, 2009)**.

The relation between amino acid levels in milk and feed

Supplementary feed and nutrition are considered one of the most important factors that play a crucial role in enhancing the production of milk and its composition. Based on the contents of AA in all feeds and milk, which are given in Table 3 and Table 5, the relations between average contents of each AA in the milk of eight broodmares and studied feeds for the lactating mares on the 2nd, 5th, 10th and 28th days of lactation are summarized in Figure 1, Figure 2 and Figure 3.

Significant relationships between all the compared data were observed; it was indicated that the deviations in levels of each amino acid in milk correlated mutually with those found in feeds on all days of lactation, with the exception of the 2nd day. All amino acid contents on the 2nd day of lactation were considerably higher than AA levels on other days; the above was due to the end of the colostral period **(Csapó et al., 2009)**. Particularly in EAA arginine, isoleucine, leucine, lysine, and phenylalanine, in NEAA Asp+Asn, Glu+Gln, proline and serine, the above tendency was pronounced significantly.

From the 5th to 28th lactation days, all amino acid contents in milk slightly decreased. It was reported that total AA content on the 8 – 45th days was twice lower than that on the 5th lactation day **(Csapó-Kiss et al., 1995)**. In contrast, this study demonstrated that on the 10th lactation day all the AA concentrations, except for EAA lysine, methionine, threonine and for NEAA glycine, increased slightly while all amino acid concentrations in feeds increased.

Methionine, lysine and phenylalanine are the most limiting crucial amino acids contained in animal feed; some feeds slightly improve their amount in milk **(Abu-Ghazaleh, Schingoethe and Hippen, 2001)**. With the exception of methionine, these AA contents were found to be adequate for the lactating mares in all the feeds. Regarding feeds during all lactation days, AA contents in feed were found to be in an adequate amount to cover daily intake of the lactating mare. As reported in the literature, by increasing the level of energy in feed and of the proportion of protein concentrate in the diet, mare's milk amount increased, but milk protein content decreased by 4 $g.L^{-1}$. Moreover, the protein supplementation in the diet showed inconsistent effects on milk protein content.

Additionally, as researchers used a supplement of protein concentrate in a hay-based diet, milk protein content

increased from 17 to 22 g.kg^{-1} **(Martuzzi and Doreau, 2006)**. Further, ratios of total EAA and NEAA content in milk to that in feed on the lactation days obeyed the following values 1:1.5, 1:2, 1:2 and 1:2.4, and 1:1.9, 1:2.5, 1:2.5 and 1:2.8, respectively. In the case of TAA content, the ratio was 1:1.7, 1:2.4, 1:2.4, and 1:2.7. On the other hand, these ratios on the 5th and 10th days of the milk period were consistent while that relating to the 28th day of lactation slightly increased. It has been reported that the ingestion of high-quality protein increases the AA concentration in mare's milk **(Glade and Luba, 1990)**. Thus, the AA concentrations in milk found in this study were significantly related to AA concentrations in the feeds.

CONCLUSION

All results of this research indicated significant relations and changes in major minerals and amino acid composition of different lactating mares' milk throughout the lactation period and in feeds used to feed the mares.

The average DM content of the feed ranged within 89.2 ±0.72 % w/w (p <0.05). Total amino acid contents in all feed generally ranged from 84.4 g.kg^{-1} to 117.0 g.kg^{-1} and the highest total EAA content was 51.7 g.kg^{-1} in 4F on the 10th day while the lowest EAA content dropped to 38.4 g.kg^{-1} in 2F on the 5th day; the major EAAs were represented by leucine and arginine, whose levels ranged within 7.3 – 10.3 g.kg^{-1} and 6.37 – 9.59 g.kg^{-1}, respectively. Methionine and histidine were found minor EAAs with levels oscillating between 2.11 – 3.05 g.kg^{-1} and 2.48 – 3.60 g.kg^{-1}, respectively. In the case of NEAA, their levels were lower compared to those of EAAs contents, which ranged between 66.8 g.kg^{-1} and 50.7 g.kg^{-1}. Glu+Gln, Asp+Asn, and proline were the major NEAAs which represented approximately 60% of TNEAA. The ratio of total EAA to NEAA ranged from 1:1.2 to 1:1.4. Generally, average total AA content in feeds for the lactating mares was found at 105.0 g.kg^{-1}.

According to results obtained, the AA content in all milk samples varied, which was caused by differences in mares and by variations throughout lactation days. The AA contents in 1P milk were three times lower (12.2 – 20.4 g.kg^{-1}) compared to other mares. With exception of 1P, total AA content in 2-8 Ps caused by breed differences ranged 21.9 – 54.6 g.kg^{-1}, 33.6 – 70.7 g.kg^{-1}, 38.1 – 71.2 g.kg^{-1}, 29.5 – 74.2 g.kg^{-1}, 52.2 – 87.1 g.kg^{-1}, 37.9 – 70.3 g.kg^{-1} and 26.4 – 64.5 g.kg^{-1}, respectively, from the 2nd to 28th day of lactation. The average decrease of total amino acid content reached 45.5% from the initial milk period to the 2nd day of the milking period. The average total AA contents caused by breed differences on the 2nd, 5th, 10th and 28th lactation days reached levels at 62.8 g.kg^{-1}, 42.8 g.kg^{-1}, 44.7 g.kg^{-1} and 44.8 g.kg^{-1}, respectively on the 2nd, 5th, 10th and 28th lactation days. During the lactation period, total AA composition did not change, but their amount significantly altered. Concerning TEAA in milk, the highest levels of EAAs were represented by arginine, leucine, and lysine, whose amounts ranged between 2.41 – 4.35 g.kg^{-1}, 3.36 – 5.59 g.kg^{-1} and 2.7 – 4.8 g.kg^{-1}, respectively while the lowest EAAs were exemplified by histidine and methionine, whose levels oscillated between 0.9 – 1.6 g.kg^{-1} and 1.2 – 2.0 g.kg^{-1}, respectively. The total NEAA content was slightly higher than that of EAA; the TNEAA to TEAA ratio was found at 1:0.9 proximately. The major NEAAs in milk were represented by Glu+Gln, Asp+Asn, and proline, ranging between 6.8 – 11.0 g.kg^{-1}, 3.2 – 5.6 g.kg$^{-1,}$ and 1.3 – 2.2 g.kg^{-1}, respectively. In contrast, levels of NEAA cysteine and glycine ranged within 0.9 – 1.5 g.kg^{-1} and 0.6 – 1.2 g.kg^{-1}, respectively.

Significant relationships between all the compared data were observed; it was indicated that the deviations in each amino acid level in milk were accordingly correlated with those found in feed on all days of lactation, except the 2nd one. Overall, the ratios of total EAA and NEAA content in milk to that in feed throughout the lactation period follow 1:1.5, 1:2, 1:2 and 1:2.4; and 1:1.9, 1:2.5, 1:2.5 and 1:2.8, respectively. In the case of TAA content, the ratio was 1:1.7, 1:2.4, 1:2.4, and 1:2.7.

Further studies on mare's digestion of proteins, levels of free amino acids, and minerals in feed and on changes in the nutrients in blood and milk of the lactating mare are suggested to clarify changes in milk composition depending on feeding systems.

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PHYTOCHEMICAL PROFILE AND BIOLOGICAL ACTIVITY OF SELECTED KIND OF MEDICINAL HERBS

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ABSTRACT

Medicinal herbs are used due to their health benefits, a special aroma, taste and are considered as one of the richest sources of bioactive compounds. The present study aimed to determine antioxidant activity (DPPH and phosphomolybdenum method), a total polyphenol (using Folin-Ciocalteu reagent), flavonoid (aluminium chloride method), phenolic acid content (using Arnov reagent), antimicrobial activity (disc diffusion method) and chemical composition (ICP-OES instrument) of medicinal herbs (ginger, comfrey, valerian, chicory, horseradish, and ramsons) grown in Slovak republic. Antioxidant activity by DPPH method ranged from 0.61 (ramsons) to 3.62 (ginger) mg TEAC per g of dry matter (TEAC – Trolox equivalent antioxidant capacity); by phosphomolybdenum method from 66.67 (valerian) to 204.14 (ginger) mg TEAC per g of dry matter. Total polyphenol content ranged from 4.37 (comfrey) to 13.19 (ramsons) GAE per g of dry matter (GAE – gallic acid equivalent); total flavonoid content from 1.07 (chicory) to 47.55 (ramsons) QE per g of dry matter (QE – quercetin equivalent) and total phenolic acid content from 0.99 (horseradish) to 9.77 (ginger) CAE per g of dry matter (CAE – caffeic acid equivalent). In a sample of ginger was detected the highest antimicrobial activity against *Bacillus cereus* CCM 7934 (5 mm). Among the mineral compounds – in all observed samples were dominated ($mg.100g^{-1}$) of potassium, phosphor, magnesium, and calcium. The amount of cadmium, chrome, and lead in observed samples was detected only in a trace amount, so our results reveal that the medicinal herbs do not represent in this study a potential health risk regarding the content of toxic elements. The consumption and using of medicinal plants as a part of the food mode of consumers due to health benefits is recommended.

Keywords: mineral compounds; antioxidant activity; polyphenols; antimicrobial activity; plants

INTRODUCTION

The use of herbal medicines is increasing steadily throughout the world as an alternative treatment for treating several health problems including civilisation diseases like cardiovascular problems, diabetes, high blood pressure and even certain types of cancer **(Kaur, Kaur and Mahajan, 2013)**. Herbs have been recognized as potential drug candidates because they possess drug-like properties **(Shakya, 2016)**. In Slovakia use of medicinal herbs is much more popular mostly in folk medicine. Unlike tablets and pills, herbal products are not regulated for purity and potency, so detection of benefits and risk is necessary.

Ginger (*Zingiber officinale* Rosco) which belongs to family *Zingiberaceae* is a plant containing a wide range of bioactive compounds especially gingerols, which are described by their pungency. The most abundant pungent component of ginger is 6-gingerol and is claimed to contain antioxidant and antimicrobial activity. Ginger has been widely used to treat nausea and vomiting; healing of wounds, cuts and has antipruritic, anticancer and anti-inflammatory activity **(Danwilai et al., 2017; Mošovská, Nováková and Kaliňák, 2015)**.

The comfrey (*Symphytum officinalis* L.) belongs to family *Boraginaceae*, is a plant that is used for therapeutic purposes mainly in traditional medicine. It is used externally for bruises, joint pains and rheumatic and also for the treatment of diseases of the gastrointestinal tract **(Nossa González, Talero Pérez and Rozo Núñez, 2016)**. The main bioactive compound of comfrey is allantoin responsible for cell division initiation and of growth of the conjunctive tissue, bones, cartilages and acceleration of wound healing **(Neagu, Roman and Radu, 2010)**.

Valerian (*Valeriana officinalis* L.) which belongs to the family *Valerianaceae* has long been used in folk medicine for the treatment of insomnia all over the world, mainly in the United States and Europe. The dominant mechanisms for the pharmacological action of valerian have been described based on their agonistic effects via gamma-aminobutyric acid, adenosine, barbiturate, and benzodiazepine receptors. Indeed, the health properties of some components present in valerian are believed to be associated with their antioxidant activities **(Sudati et al., 2009)**.

Chicory (*Cichorium intybus* L.) which belongs to family *Asteraceae* is widely distributed in Asia and Europe. All parts of this plant expose medicinal properties due to the presence of several bioactive compounds such as inulin, lactones, coumarins, vitamins, natural colorants, sterols, polyphenols, saponins and tannins **(Abbas et al., 2015)**. Preparations from chicory exhibiting antioxidant, immunomodulating, anti-inflammatory activity due to the presence of cichoric acid as a specific active compound **(Ahamad et al., 2015)**.

Horseradish (*Armoracia rusticana* Gaertn) which belongs to the family *Brassicaceae* is used freshly grated in gastronomy as an important ingredient for meat and fish products or into sauces and salads. The main bioactive substances of horseradish are glucosinolates that provide the characteristic flavour, aroma and are also responsible for health-promoting effects such as anti-cancer properties, antioxidant and antimicrobial activity **(Calabrone et al., 2015)**.

Ramsons (*Allium ursinum* L.) which belongs to the family *Amaryllidaceae* has been used for centuries in alternative medicine. Mostly of studies based on its composition and biological activity are fairly recent and scarce. The main bioactive compounds of ramsons are sulfur-containing compounds that are responsible for distinct garlic-like scent and also for health benefits: digestive stimulant, antimicrobial agent, removing toxins from the body, and to prevent cardiovascular disorders **(Sobolewska, Podolak and Makowska-Was, 2015)**.

Scientific hypothesis

Medicinal herbs are a good source of bioactive compounds with antioxidant and antimicrobial activity. In some cases that can also be poisonous due to the heavy metals content. In our study we assumed a high biological activity and low level of hazard mineral compounds, providing the possibility of using these herbs in different kinds of industry.

MATERIAL AND METHODOLOGY

Biological material

The medicinal herbs were collected from nature and gardens in Slovakia (the Year 2018; locality Runina; 560 m.a.s.l.): *Zingiber officinale* Rosco – rhizome, *Symphytum officinalis* L. – root, *Valeriana officinalis* L. – root, *Cichorium intybus* L. – root, *Armoracia rusticana* Gaertn – root and *Allium ursinum* L. – leaves. Before the analysis samples were pulverized in the mortar.

Sample preparation

The sample – 0.2 g was extracted with 20 mL of 80% ethanol for 2 hours. After centrifugation at 4000 rpm (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for measurement (antioxidant activity, polyphenols, flavonoids, phenolic acids). All analyses were realized in triplicate.

Chemicals

All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

Antioxidant activity

DPPH method – radical scavenging activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(Sánchéz-Moreno, Larrauri and Saura-Calixto, 1998)** with slight modification. The extracts (0.4 mL) were mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). The absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 1000 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Phosphomolybdenum method – reducing power

Reducing the power of samples was determined by the method of **Prieto, Pineda and Aguilar (1999)** with slight modification. The mixture of the sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90 °C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 1000 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Total polyphenol content

The total polyphenol content of samples was measured by the method of **Singleton and Rossi (1965)** using Folin-Ciocalteu reagent. An amount of 0.1 mL of each sample was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate, and 8.8 mL of distilled water. After 30 min. in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25 – 300 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard and the results were expressed in $mg.g^{-1}$ gallic acid equivalents.

Total flavonoid content

The total flavonoid content of samples was determined using the modified method of **Willett (2002)**. An amount of 0.5 mL of each sample was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M potassium acetate, and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (0.5 – 20 $mg.L^{-1}$; $R^2 = 0.989$) was used as the standard and the results were expressed in $mg.g^{-1}$ quercetin equivalents.

The total phenolic acid content

The total phenolic acid content of samples was determined using the method of **Farmakopea Polska (1999)**. An amount of 0.5 mL of each sample was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnov reagent (10% $NaNO_2$ +10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1 – 200 $mg.L^{-1}$; $R^2 = 0.999$) was used as a standard and the results were expressed in $mg.g^{-1}$ caffeic acid equivalent.

Microbial strains

Six strains of microorganisms were tested in this study. Gram-negative bacteria: *Escherichia coli* CCM 2024, *Citrobacter freundii* CCM 7187, *Yersinia enterocolitica* CCM 7204 and Gram-positive bacteria: *Bacillus cereus* CCM 7934, *Staphylococcus aureus* subsp. *aureus* CCM 2461, *Listeria monocytogenes* CCM 4699. All tested strains were collected from the Czech Collection of microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37 °C.

Antimicrobial activity

The antimicrobial activity of each extract was determined by a disc diffusion method. Briefly, 100 μL of the test bacteria were grown in 10 mL of fresh media until they reached a count of approximately 10^5 cells per mL. Then 100 μL of the microbial suspension was spread onto Mueller Hinton agar plates. The extracts were tested using 6 mm sterilized filter paper discs. The diameters of the inhibition zones were measured in millimeters. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate. Filter discs impregnated with 10 μL of distilled water were used as a negative control.

Chemical (mineral compounds) composition

The amount of mineral elements was analysed by the ICP-OES method (ICP-OES spectrophotometer, Thermo iCAP Dual 6500, USA) – the samples (0.2 g) were subjected to mineralization under high pressure, in HNO_3 65%, super pure. The samples were weighed and placed in Teflon vessels which were then filled with 8 mL of nitric acid and sealed tightly. For each group of nine samples, during the microwave dissolution process, the rotor of the digestion system was additionally filled with a blank sample comprising 8 mL of nitric acid alone. The samples were digested for one hour, with the applied algorithm of temperature increase as specified for biological samples, without exceeding 200 °C. This was carried out using Ethos One microwave digestion system from Milestone. The vessels were opened after the mineralization process had been completed and the samples with acid had been brought to room temperature. The samples were cooled down to room temperature and supplemented with water to the volume of 50 mL. The obtained detection threshold for each element was not lower than 0.01 $mg.kg^{-1}$ (with the assumed detection capacity of the measuring apparatus at a level exceeding 1 ppb). The measurements were performed with ICP-OES spectrometer, Thermo iCAP Dual 6500 with horizontal plasma, and the capacity of detection along and across plasma flame (Radial and Axial). Before measuring each batch of samples the method was calibrated with the use of certified Merck models. The measurement result for each element was compensated to account for the measurement of elements in the blank sample. In each case, a 3-point calibration curve was used for each element, with optics correction applying the method of internal models, in the form of yttrium and ytterbium ions, at the concentrations of 2 $mg.L^{-1}$ and 5 $mg.L^{-1}$, respectively.

Statistical analysis

All experiments were carried out in triplicate and the results reported are expressed as means with standard deviations. The experimental data were subjected to the analysis of variance (Duncan's test), at the confidence level of 0.05, using the **SAS 2009** (USA) software.

RESULTS AND DISCUSSION

Antioxidant activity

Antioxidant activity (Table 1) of analysed samples tested by the DPPH method ranged from 0.61 to 3.62 mg $TEAC.g^{-1}$. The highest value was determined in the sample of ginger, following by a sample of chicory and valerian. Antioxidant activity tested by the phosphomolybdenum method (Table 1) had a similar tendency – the highest value was measured in a sample of ginger (204.14 mg $TEAC.g^{-1}$). Similar value determined in ginger by reducing power method **Akinola, Ahmad and Maziah (2014)** – 266.95 mg $TEAC.g^{-1}$ The strong activity by DPPH, ABTS, and FRAP method of ginger was observed in the study of **Mustafa et al. (2019)**. This authors also reported that activity is influenced by the technological process – the best activity in FRAP and ABTS method was found in ginger sun-dried, while in the DPPH method the strongest activity was found in fresh ginger. Antioxidant activity of valerian was observed in the study of **Pilerood and Prakash (2014)**. These authors tested ethanol, methanol, acetone, 80% methanol, and 80% ethanolic extracts of valerian. The best activity determined by using 80% of ethanol – this extraction solution was also used in our study. In a sample of ramsons (Table 1) by the DPPH method was evaluated the lowest activity, whereas by the phosphomolybdenum method the activity was very strong. This result can be explained by the mechanism of action – in the DPPH method antioxidants from the sample act as scavengers of radical, whereas in phosphomolybdenum method antioxidants from the sample act as reductant agent. In the study of **Putnoky, Caunii and Butnariu (2013)** are described that the S–oxyde (+/−) of S–2–propenyl cysteine and the S–oxide of S–alkenyl cysteine, located in the cytoplasm and the enzyme alliinase located in the vacuole is the main bioactive compounds which bind reactive free radicals and act as reductant agent.

Total polyphenol, flavonoid, and phenolic acid content

Total polyphenols (Table 1) in tested samples ranged from 4.37 to 13.19 mg $GAE.g^{-1}$. The highest value was found in the sample of ramsons following by ginger and valerian. **Lachowicz et al. (2017)** determined in ramsons values ranged from 6.5 (bulbs) to 42.5 (leaves) mg $GAE.100g^{-1}$ fresh matter which is lower values compared to our study. **Pejatović, Samardžić and Krivokapić (2017)** determined values ranged from 13.05 to 18.33 mg $GAE.g^{-1}$, which is comparable with our findings. **Qadir et al. (2017)** in ginger extract found total polyphenols in the amount of 98.37 mg $GAE.100g^{-1}$, which is a lower level to compare with our results. These authors also published that 80% of ethanol is discovered more effective for the recovery of antioxidants from herbal plants to compare with methanol and acetone.

Table 1 Antioxidant activity, total polyphenol, flavonoid and phenolic acid content in tested samples.

Sample	DPPH (mg TEAC.g^{-1})	PM (mg TEAC.g^{-1})	TPC (mg GAE.g^{-1})	TFC (mg QE.g^{-1})	TPAC (mg CAE.g^{-1})
Ginger (*Zingiber officinale* Rosco)	3.62 ±0.08a	204.14 ±2.99a	11.91 ±0.11a	8.62 ±1.11b	9.77 ±0.09a
Horseradish (*Armoracia rusticana* Gaertn)	1.01 ±0.11e	73.94 ±3.08b	4.09 ±1.04b	1.96 ±0.07c	0.99 ±0.07f
Comfrey (*Symphytum officinalis* L.)	1.16 ±0.03d	95.09 ±3.03c	4.37 ±0.06b	2.21 ±0.11c	3.31 ±0.09c
Ramsons (*Allium ursinum* L.)	0.61 ±0.02f	169.18 ±2.44b	13.19 ±1.72a	47.55 ±2.31a	8.31 ±0.31b
Cichory (*Cichorium intybus* L.)	2.79 ±0.04b	80.31 ±2.41d	5.39 ±0.83b	1.07 ±0.04c	1.79 ±0.01e
Valerian (*Valeriana officinalis* L.)	2.52 ±0.07c	66.67 ±3.11f	5.53 ±0.14b	1.67 ±0.09c	2.61 ±0.07d

Note: DPPH – radical scavenging activity; PM – phosphomolybdenum method; TPC – total polyphenol content; TFC – total flavonoid content; TPAC – total phenolic acid content; TEAC – Trolox equivalent antioxidant capacity; GAE – gallic acid equivalent; QE – quercetin equivalent; CAE – caffeic acid equivalent; mean ±standard deviation; different letters in column denote mean values that statistically differ one from another.

Table 2 Antimicrobial activity of tested samples determined by disc diffusion method.

Sample	*E.coli* CCM 2024 (mm)	*C. freundii* CCM 7187 (mm)	*Y. enterocolitica* CCM 7204 (mm)	*B. cereus* CCM 7934 (mm)	*S. aureus* CCM 2461 (mm)	*L. monocytogenes* CCM 4699 (mm)
Ginger (*Zingiber officinale* Rosco)	-	-	1.00 ±0.07b	5.00 ±0.39a	-	1.00 ±0.02c
Horseradish (*Armoracia rusticana* Gaertn)	-	-	-	1.33 ±0.11c	-	1.33 ±0.05c
Comfrey (*Symphytum officinalis* L.)	1.00 ±0.01c	1.33 ±0.14b	1.00 ±0.06b	-	-	2.66 ±0.71a
Ramsons (*Allium ursinum* L.)	2.00 ±0.11b	2 ±0.11a	2.33 ±0.01a	-	2.00 ±0.02a	1.00 ±0.11c
Cichory (*Cichorium intybus* L.)	3.33 ±0.21a	-	1.00 ±0.01b	2.33 ±0.31b	2.00 ±0.03a	2.00 ±0.12b
Valerian (*Valeriana officinalis* L.)	-	1.00 ±0.02c	1.00 ±0.02b	2.33 ±0.24b	2.00 ±0.01a	1.00 ±0.09c

Note: mm - millimetre; mean ±standard deviation; different letters in column denote mean values that statistically differ one from another.

Table 3 Mineral compounds composition in tested samples.

Parameter (mg.100g^{-1})	Ginger (*Zingiber officinale* Rosco)	Horseradish (*Armoracia rusticana* Gaertn)	Comfrey (*Symphytum officinalis* L.)	Ramsons (*Allium ursinum* L.)	Cichory (*Cichorium intybus* L.)	Valerian (*Valeriana officinalis* L.)
Aluminium (Al)	11.50 ±2.04e	32.00 ±2.22d	55.00 ±1.42b	9.68 ±1.75e	41.91 ±1.12c	133 ±2.58a
Cadmium (Cd)	0.04 ±0.01a	0.03 ±0.01ab	0.02 ±0.01bc	0.01 ±0.01c	0.02 ±0.01bc	0.02 ±0.01bc
Chrome (Cr)	0.09 ±0.01b	0.19 ±0.05b	0.70 ±0.02b	0.35 ±0.02b	0.70 ±0.01b	2.85 ±1.17a
Calcium (Ca)	233 ±3.21f	365 ±3.45c	375 ±3.33b	702 ±2.59a	252 ±2.63e	321 ±2.41d
Copper (Cu)	0.76 ±0.13c	0.33 ±0.05d	1.31 ±0.12a	0.46 ±0.11d	0.90 ±0.01c	1.07 ±0.09b
Iron (Fe)	10.9 ±0.69e	22.9 ±1.14d	57.6 ±2.22b	12.3 ±1.24e	49.1 ±3.32c	189 ±1.52a
Potassium (K)	3772 ±2.45a	1961 ±18.80c	1778 ±3.58d	2655 ±10.81b	1234 ±3.14f	1698 ±4.48e
Magnesium (Mg)	390 ±1.79a	130 ±1.09e	118 ±4.21f	251 ±3.75c	182 ±1.97d	275 ±3.24b
Manganese (Mn)	182 ±1.24a	130 ±1.09b	4.02 ±0.11de	6.00 ±1.98d	2.72 ±0.58f	16.10 ±0.94c
Sodium (Na)	79.4 ±2.09c	6.23 ±1.96e	419 ±2.74b	3.24 ±1.11e	708 ±2.77a	20.3 ±1.12d
Phosphorus (P)	376 ±0.73b	196 ±2.80f	200 ±1.11e	391 ±2.13a	282 ±3.11d	343 ±1.14c
Lead (Pb)	0.09 ±0.02b	0.04 ±0.01c	0.08 ±0.01b	0.02 ±0.01c	0.08 ±0.02b	0.41 ±0.02a
Sulphur (S)	178 ±3.11c	615 ±3.56b	84.8 ±1.23f	932 ±3.21a	119 ±1.73e	0.41 ±1.11bc
Strontium (Sr)	1.93 ±0.09b	2.38 ±1.28b	1.53 ±0.09bc	0.30 ±0.10c	4.61 ±1.04a	1.44 ±0.09bc
Zinc (Zn)	5.06 ±1.79a	3.77 ±0.58ab	1.17 ±0.07c	1.34 ±0.08c	0.65 ±0.08c	2.99 ±0.07b

Note: mean ±standard deviation; different letters in column denote mean values that statistically differ one from another.

Total flavonoids (Table 1) in tested samples ranged from 1.07 to 47.55 mg QE.g^{-1}. The best value was measured in a sample of ramsons following by a sample of ginger and comfrey. **Pejatović, Samardžić and Krivokapić (2017)** determined in ramsons leaves extract values ranged from 13.75 to 20.00 mg QE.g^{-1}. **Ghasemzadeh, Jaafar and Rahmat (2010)** in a sample of ginger extract determined total flavonoids in amount ranged from 3.66 to 4.21 mg QE.g^{-1}, which are lower values to compare with our results. These authors by HPLC method determined that in ginger rhizomes the most abundant flavonoid is quercetin. In valerian the most abundant flavonoids are 6-methylapigenin and hesperidin, which are responsible for the sedative and sleep-enhancing properties of valerian root **(Marder et al., 2003)**.

Total phenolic acid content (Table 1) ranged from 0.99 to 9.77 mg CAE.g^{-1}. The highest value was determined in a sample of ginger following by a sample of ramsons and comfrey. **Fahmi et al. (2019)** published that chlorogenic acid (63.85 ppm) and hesperidin (156.91 ppm) are among the major phenolic and flavonoid constituents in dry ginger. In comfrey **Sowa et al. (2017)** by HPLC-DAD method found rosmarinic, *p*-hydroxybenzoic, caffeic, chlorogenic, and *p*-coumaric acid.

Antimicrobial activity

In a sample of ginger (Table 2) was observed activity to inhibit *B. cereus* CCM 7934, *Y. enterocolitica* CCM 7204 and *L. monocytogenes* CCM 4699. In the study of **Nas, Ali and Ahmad (2018)** *E. coli* were found to be the highest susceptible organisms with an average zone of inhibition of 13.6 mm, followed by the *Shigella spp.* (13.3 mm), *Salmonella typhi* (12.7 mm), *Staphylococcus aureus* (12.5 mm), *Pseudomonas aeruginosa* (10.8 mm) while the least average zone of inhibition is shown by *Klebsiella pneumoniae* (9.2 mm).

In the sample of horseradish (Table 2) was detected low potential to inhibit growing of *B. cereus* CCM 7934 and *L. monocytogenes* CCM 4699. In comfrey extract (Table 2) was detected activity to inhibit *E.coli* CCM 2024, *C. freundii* CCM 7187, *Y. enterocolitica* CCM 7204 and *L. monocytogenes* CCM 4699. The antibacterial activity of the root extract of *Symphytum officinale* was tested by the disc diffusion method in the study of **Sumathi, Kumar and Bai (2011)** and it was found that methanol extract showed maximum inhibitory effect against the *Proteus vulgaris* and *Staphylococcus aureus*.

In ramsons sample (Table 2) was observed slight activity to inhibit *E. coli* CCM 2024, *C. freundii* CCM 7187, *Y. enterocolitica* CCM 7204, *S. aureus* subsp. *aureus* CCM 2461 and *L. monocytogenes* CCM 4699. **Krivokapic et al. (2018)** reported that the antimicrobial activity of the plant extract is influenced by the extraction solvent. A methanol extract of ramsons has been shown to exhibits antimicrobial activity against bacteria including *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli, Proteus mirabilis, Salmonella enteritidis*. On the other hand, water extract was efficient only against *Bacillus subtilis*. Chicory extract in our study showed slight activity to inhibit *E. coli* CCM 2024, *B. cereus* CCM 7934, *Y. enterocolitica* CCM 7204, *S. aureus* subsp. *aureus* CCM 2461 and *L. monocytogenes* CCM 4699. In the study, **Liu et al. (2013)** were observed several extracts of chicory which displayed activities against *Escherichia coli, Staphylococcus aureus, Bacillus thuringiensis, Bacillus subtilis*, and *Salmonella typhi*. These authors also found that the best method for the chicory extract yield with antimicrobial activity was a combination of 70% ethanol v/v, 24-h impregnation time, 3 sonication rounds, and 300-W ultrasonic input power. In valerian extract (Table 2) was observed potential to inhibit the growth of *C. freundii* CCM 7187, *Y. enterocolitica* CCM 7204, *B. cereus* CCM 7934, *S. aureus* subsp. *aureus* CCM 2461 and *L. monocytogenes* CCM 4699.

Chemical composition

The results of mineral compounds composition are presented in Table 3. In a sample of ginger was determined potassium as the dominant compound following by magnesium and phosphorus. Very interesting was the amount of zinc which was the highest in this sample compared to other tested samples. According to **Chasapis et al. (2012)** zinc is one of the most important trace elements in the human body, with three major biological roles: catalyst, structural, and regulatory ion. Zinc has an important effect on homeostasis, immune function, oxidative stress, apoptosis, aging, and significant disorders of great public health interest are associated with zinc.

In samples of horseradish were dominant potassium, sulphur, calcium and phosphorus. The amount of sulphur was higher to compare with ginger, valerian, chicory, and comfrey. A higher amount of sulphur can be explained as the fact that horseradish is rich for glucosinolates. Glucosinolates are N-hydroxy-sulfates with a highly variable side chain and a sulfur-linked *beta*-d-glucopyranose. These compounds are the precursor molecules of the biologically active isothiocyanates components **(Bertóti et al., 2019)**.

In a sample of comfrey dominant compounds was potassium, following by sodium, phosphorus, and calcium. This sample was determined the highest value of copper, which is an essential mineral for human health but at the same time can be toxic, depending upon the amounts of ingested. Copper is associated with bone health, immune function, and increased frequency of infections, cardiovascular risk, and alterations in cholesterol metabolism **(Araya, Manuel and Fernando, 2007)**.

In the sample of ramsons was determined potassium as dominant compounds following by the sulphur, calcium, and magnesium. The amount of sulphur was the highest to compare with the other analysed samples. It is not surprising whereas the dominant bioactive compounds of this plant are based of sulphur (allicin).

In the sample of chicory, the main mineral compounds were potassium, sodium, magnesium and sulphur. The same tendency was observed in the study of **Mona, Wafaa and Elgindy (2009)** in which also authors determined higher content of iron in chicory root, which is comparable with our findings.

In the sample of valerian was determined potassium as dominant mineral compounds following by calcium and magnesium. In this sample was found the highest value of aluminium, this amount can be evaluated as a safe because according to Food Safety Authority **(Stahl et al., 2017)** the tolerable weekly intake of aluminium is 1 mg.kg^{-1} body weight for all groups of people.

Valerian root is used mainly in folk medicine to prepare a decoction to treat psychical problems and usually is not consumed as a vegetable. Generally, the amount of heavy metals in observed samples was detected only in a trace amount, so our results reveal that the medicinal herbs do not represent in this study a potential health risk regarding the content of toxic elements.

CONCLUSION

Medicinal herbs are a very important part of the industry, especially pharmacy and medicine. Nowadays these plants start to be very popular also in gastronomy and food technology. They are a good source of specific bioactive compounds, but their quality can be endangered due the to higher level of heavy metals. In our study, the highest antioxidant activity was determined in ginger (3.62 and 204.14 mg TEAC.g^{-1}). In the sample of ramsons was detected the highest value of phenolics (13.19 mg GAE.g^{-1}; 47.55 mg QE.g^{-1}). The wide spectrum of mineral compounds was generally found in all observed samples, while the amount of heavy metals was detected only in a trace amount.

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EFFECT OF GERMINATED WHEAT (*TRITICUM AESTIVUM*) ON CHEMICAL, AMINO ACID AND ORGANOLEPTIC PROPERTIES OF MEAT PATE

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ABSTRACT

Germinated cereal crops are widely used in the technology of meat products, as they contain a significant amount of vitamins, minerals and protein. This study presents the formulation and processing technology of meat pate with the addition of wheat (*Triticum aestivum*) germ. Three treatments of 10, 15 and 20% of germinated wheat (GW) were prepared. Wheat sprouts were crushed and mixed for 6 minutes in a meat mixer together with broth, oat flour, and spices to obtain a smooth mass. This was mixed with minced meat and grinded on a cutter to obtain a more uniform finished paste that was dosed into lamister or tin containers, sterilized and stored for 2 years. This was followed by the determination of its proximate composition, water-binding capacity, and sensory analysis. Results show that the meat pate with 10% of GW in comparison with the control, contains more protein, is more nutritious with a lower fat content of 6.8%, and a lower carbohydrate content of 11.3%. The results of the organoleptic evaluation showed that the highest average score was for the meat pate with a recipe that contains 10% of germinated wheat, and the lowest was for the meat pate containing 20% of germinated wheat. Meat pate containing 10% of germinated wheat has a higher water-holding capacity and the optimal pH value. This study suggests that wheat can be used in appropriate formulation to improve the organoleptic quality of meat pate.

Keywords: meat pate; wheat; germination; water-holding capacity

INTRODUCTION

The modern approach of nutrition science applies high requirements to food products quality, taking into account not only its energy value but also the presence of components necessary for human health (a complex of biologically active substances, dietary fiber, pectin, organic acids, minerals, etc.) possessing immunostimulating, radioprotective, prophylactic and healing properties **(Kakimov et al., 2017; Kolbábek et al., 2019)**. Due to the deficiency of meat, meat industry experts are developing new methods of production by replacing meat with protein-containing raw materials and protein supplements that are close in quality to meat protein **(Kenenbay et al., 2017; Momchilova et al., 2019)**. Recently, there has been a steady trend in the development and production of combined meat products, where along with meat, the plant raw materials sampled from the local region are used to regulate the protein, lipid, amino acid, fatty acid, carbohydrate, mineral and vitamin composition of the food product **(Arihara, 2006; Kassenov et al., 2019)**. The production of combined meat products based on plant materials is one of the promising areas for the creation of food products for the medical and preventive purposes of the modern food industry (**Nishanova et al., 2020**). Pate is a meat product made of thermally processed ingredients with spreading consistency. The classic pate recipe includes liver, offal, meat, animal fats (lard, poultry fat, and butter), pepper, spices, and salt **(Okuskhanova et al., 2016; Smolnikova et al., 2019)**. The goal of this study was to evaluate the nutritive value of meat pate enriched with germinated wheat as a contribution towards the improvement of the organoleptic quality of this delicacy.

Scientific hypothesis

The scientific hypothesis of this work is the creation of low-waste, resource-saving technologies of new kinds of pate products, using low-grade meat raw materials and wheat sprouts, allowing to increase biological value (chemical, amino acid composition), physico-chemical and organoleptic characteristics of meat pate in a directed way.

MATERIALS AND METHODS

Materials: The technological process of production consists of the following operations

The meat trimmings from the head of cattle were washed and cut into small pieces. Then the pieces of meat trimmings were cooked for 1 – 1.5 hours. After cooking it was weighed and crushed in a meat grinder with a diameter of the plate holes (2 – 3) mm. Onions were peeled, washed, chopped

into cubes, sautéed in butter for 10 – 15 minutes, weighed, then minced in a meat grinder (plate with holes of diameter 2 – 3 mm). Wheat sprouts were crushed and the crushed components were mixed in a meat mixer. Broth, oat flour, spices were added and mixed into a smooth mass. The mixing time was 6 minutes. The minced meat was grinded on
a cutter to obtain a more uniform structure. The finished paste was dosed into a lamister container weighing 200 grams or a tin container weighing 360 grams. Lamister is a combined material that consists of foil and polypropylene film, interconnected by an adhesive layer. The pate was sterilized using the sterilization formula (20 – 40 – 20)/120, 150 – 200 kPa at a temperature of 120 ºC for 20 minutes. The pate was stored at a temperature of 18 – 20 °C with a relative humidity of 80% for 2 years. The technological scheme is presented in Figure 1.

Determination of proximate composition

The determination of the chemical composition of meat was based on the evaluation of the following constituents: moisture, fat, ash, and protein. The methods were performed as described by **Amirkhanov et al. (2017)**. To determine water content, a 2 – 3 g aliquot of each sample of meat was weighed to the nearest 0.001 g using a Mettler Toledo electronic balance (Greifensee, Switzerland) and placed into a metallic cup (IngoLab, Moscow. Russia). It was then dried for 1 h in a drying oven (SNOL 67/350; Umega, Utena, Latvia) at 150 °C. The moisture content was calculated using Equation 1, according to the **GOST 9793-74 (2010)** and **GOST R 51479-99 (2010)** standards.

$$x_1 = (m_1 - m_2) \cdot 100/(m_1 - m) \quad (1)$$

Where: x_1 is the moisture content (%), m_1 is the weight of the sample with a cup before drying (g), m_2 is the weight of the sample with the cup after drying (g), m is the weight of the cup alone (g).

After determining the moisture, each dried sample was moved to a glass cup. Then, 15 mL of ethyl ether (100% chemically pure; Skat, Almaty, Kazakhstan) was poured into the glass cup, and the contents were mixed for 3 – 4 min. During the extraction process, the organic fraction containing the fat residues was poured out and replaced with fresh ethyl ether. After 4 – 5 repetitions, the residual ethyl ether was evaporated at room temperature. The metallic cup containing the fat-depleted sample was dried at 105 °C for 10 min. The fat content was calculated according to the **GOST 23042-86 (2010)** standard using Equation 2.

$$x_2 = (m_1 - m_2) \cdot 100/m_0 \quad (2)$$

Where: x_2 is the fat content (%), m_1 is the weight of the cup and dry sample before extraction (g), m_2 is the weight of the cup, and sample after extraction (g), m_0 is the weight of the cup alone (g).

To obtain the ash content, the sample from which the fat was extracted was placed into a weighed and preheated (to 150 °C) crucible (50 cm^3; Mankor, Kyiv, Ukraine). Then, 1 mL of magnesium acetate (98% purity; Labofarma, Almaty, Kazakhstan) was added to the crucible and burned on an electric hot plate. After that, it was placed into a muffle furnace set at 500 – 600 °C (SNOL 7.2/1100; Umega) for 30 min. The ash content was calculated using Equation 3.

$$x_3 = (m_1 - m_2) \cdot 100/m_0 \quad (3)$$

Where: x_3 is the ash content (%), m_1 is the weight of the ash (in g), m_2 is the weight of the magnesium oxide obtained after mineralization of the magnesium acetate (g), and m_0 is the weight of the sample alone (g).

Protein content was assayed according to the **GOST 25011-81 (2010)** standard and calculated using Equation 4.

$$x = 100 - (x_1 + x_2 + x_3) \quad (4)$$

Where: x is the protein content (%), x_1 is the moisture content (%), x_2 is the fat content (%), and x_3 is the ash content (%).

Water-binding Capacity

The method used to determine the water-binding capacity (WBC) of the samples was based on exudation of moisture to a filter paper by the application of pressure. The moisture absorbed by the filter paper is evaluated based on the spot area on the filter paper. Specifically, for each sample, 0.3 g of minced meat was placed on a 15 – 20 mm diameter disk plate on a Mettler Toledo electronic balance, (Mettler Toledo, Switzerland). The meat was then transferred onto an ash-free filter (Munktell Filter AB, Sweden) and placed on a glass or plexiglass plate. The sample was covered with the same filter before a 1 kg load was carefully placed on top of the meat. The weight was left for 10 min. Once removed, the top filter was pulled off and bound water was calculated, as described below (see Equation 1 and 2). The filter was scanned using an Xpress M2070 scanner (SAMSUNG, Japan) after the contour of the wet spot was traced on the filter. The area was calculated using the Compas-3D V-10 software **(Kabulov et al., 2014; Okuskhanova et al. 2017)**

$$X_1 = (A\text{-}8.4B) \cdot 100/m_0, \quad (1)$$
$$X_2 = (A\text{-}8.4B) \cdot 100/A; \quad (2)$$

Where: X_1 is bound water content (expressed as % of meat), X_2 is bound water content (expressed as % to total water), B is a wet spot area (cm^2), m_0 is sample weight (mg), A is the total content of moisture in the sample (mg).

Sensory analysis

Sensory evaluation was done by a panel of twelve (12) skilled persons (aged 23 – 58). In case of defects in flavor and aroma (inadequate pronounced flavor, weedy flavor, and slightly acid flavor), consistency and structure, color, and packaging, the score mark were reduced for each defect according to the special sensory evaluation scale. The evaluation scale ranged from 1 to 5 points, where 1 – Unliked extremely; 5 – Liked extremely.

Table 1 Chemical composition of pate with germinated wheat grain.

Index	Pate with 10% of GW	Pate with 15% of GW	Pate with 20% of GW	Control beef pate "Kubley"
Protein (%)	14.36 ±0.30^{a}	12.60 ±0.28^{c}	13.20 ±0.31^{bc}	14.00 ±0.21^{ab}
Fat (%)	6.80 ±0.20^{b}	7.77 ±0.21^{a}	5.50 ±0.11^{c}	7.2 ±0.27^{ab}
Carbohydrate (%)	11.30 ±0.26^{b}	11.85 ±0.21^{b}	13.35 ±0.48^{a}	11.5 ±0.36^{b}
Water (%)	66.44 ±1.31^{a}	66.78 ±1.21^{a}	67.10 ±1.65^{a}	72.4 ±2.18^{a}
Ash (%)	1.10 ±0.03^{b}	1.00 ±0.01^{b}	0.85 ±0.02^{c}	1.30 ±0.04^{a}
Calorie value, kCal	161.0	163.0	152.3	154.3

Note: $^{a, b, c, d}$ means values within the same row with different superscripts significantly differ (p <0.05).

Table 2 Physico-chemical characteristics of pate with GW.

Index	Pate with 10% of GW	Pate with 15% of GW	Pate with 20% of GW	Control beef pate "Kubley"
Salt weight percent (%)	1.0	1.2	0.9	1.2
pH	6.7	6.9	6.85	6.7
Water-binding capacity (%)	73	71	68	70

Table 3 Organoleptic characteristics of meat pate.

Index	Pate with 10% of GW	Pate with 15% of GW	Pate with 20% of GW
Appearance	4.8	4.6	4.5
Color	4.9	4.7	4.8
Flavor	5.0	4.7	4.6
Taste	4.8	4.6	4.6
Consistency	5.0	4.7	4.6
Average score	4.9	4.66	4.62

Table 4 Amino acid composition of meat pate in g.100g^{-1}.

Amino acid	Pate with 10% of GW	Pate with 15% of GW	Pate with 20% of GW	Control beef pate "Kubley"
Essential	7.742	7.379	7.086	4.3549
Threonine	0.961	0.922	0.883	0.4631
Isoleucine	1.064	1.021	0.977	0.5295
Lysine	1.969	1.873	1.777	0.8087
Methionine	0.588	0.561	0.535	0.2456
Cysteine	0.247	0.241	0.235	0.182
Phenylalanine	0.863	0.834	0.806	0.5285
Valine	0.838	0.806	0.775	0.7024
Leucine	1.212	1.121	1.098	0.8951
Non-essential	10.612	10.222	9.832	4.2042
Tyrosine	1.116	1.074	1.032	0.4122
Arginine	1.483	1.424	1.365	0.7394
Histidine	0.821	0.786	0.751	0.4719
Alanine	1.299	1.243	1.187	0.5823
Aspartic acid	2.088	1.997	1.907	0.7988
Glutamic acid	3.805	3.698	3.59	1.1996
Total	18.354	17.601	16.918	8.5591

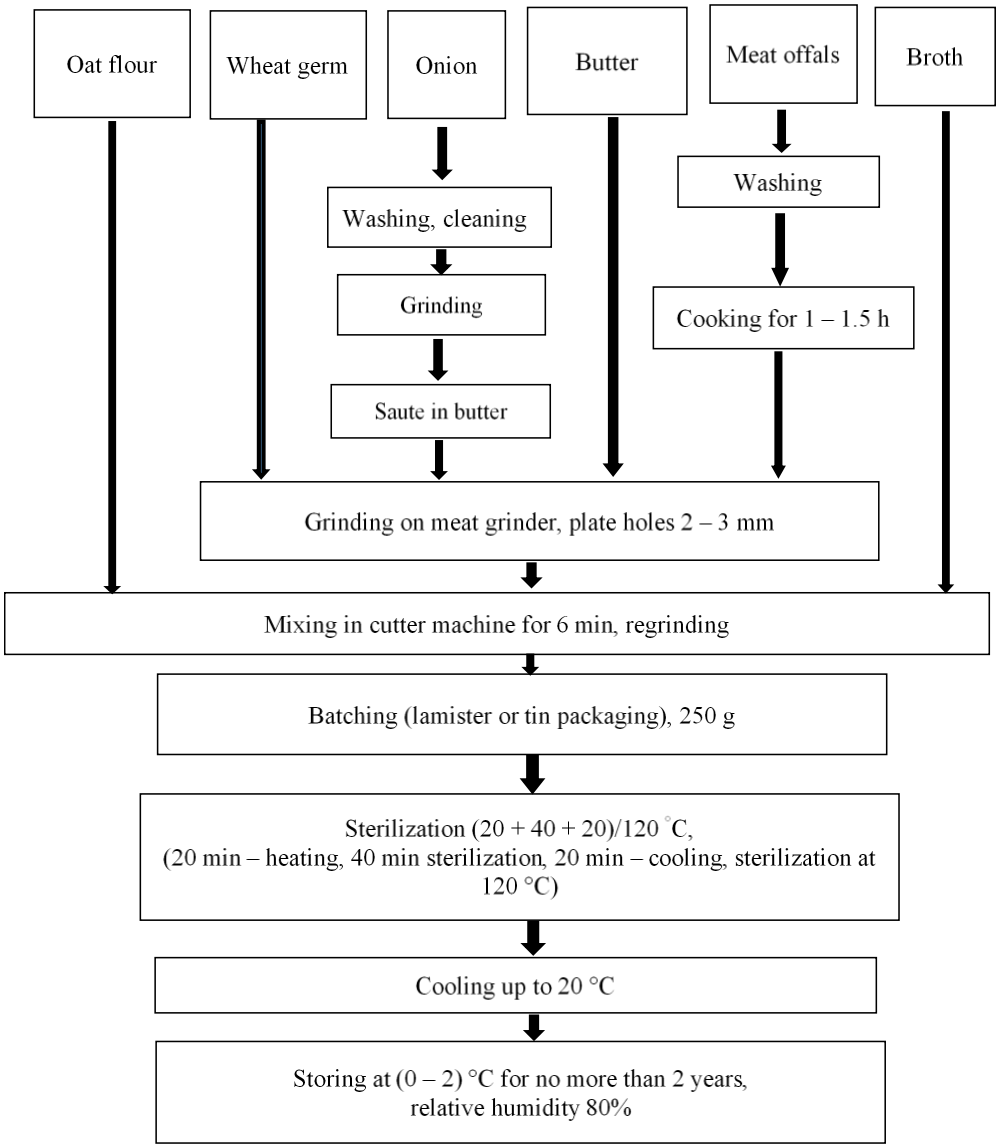

Figure 1 Meat pate processing flow chart.

Statistical Analysis

Statistical analysis was performed using Statistica 12.0 (STATISTICA, 2014; StatSoft Inc., Tulsa, OK, USA). The differences between samples were evaluated using the ANOVA method. The differences were considered to be statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Previous studies revealed that meat trimmings from heads of cattle have lower vitamin and microelement contents compared to beef **(Berdutina, 2000; Bondarenko and Elizarova, 2014; Makangali et al., 2018; Verma et al., 2008)**. At the same time, it has a sufficient level of protein content with a higher total content of essential amino acids in comparison with beef – the content of essential amino acids in beef is 37.07 g.100g^{-1} of protein, and in the head of cattle – 38.66 g.100g^{-1} of protein. The chemical composition of meat pates is shown in table 1 in comparison with the control. The obtained results (Table 1) show that meat pate with 10% of GW in comparison with the control, contains more protein and is more nutritious with a lower fat content of 6.8% and a lower carbohydrate content of 11.3%. **Gorlov et al. (2019)** also used germinated lentils (15%) and carrot (15%) in meat pate formulation. In terms of chemical composition, the pate in their study contained more protein 17.44% and lower fat (4.52%) compared with our study. However, the carbohydrates and calorie values were the same. **Lyakh et al. (2016)** proposed an optimal formulation of pate, with a ratio of components: 50% of mutton and 50% of horsemeat with the replacement of meat by dill and addition of 2 g "Policorbovit-95". The sample with replacement of 10% of meat with dill had the best organoleptic indicators. The content of protein (17.8 %) and fat (12.56 %) considerably differs from our results.

Chizhikova et al. (2017) studied the chemical composition of minced meat with the addition of 26% germinated wheat and found that the protein content was 15.8%, fat 12.5%, and ash 1.16%. At the same time, the authors noted that the addition of such amounts of germinated wheat did not worsen the organoleptic properties of minced meat. **Martemyanova and Yasakov (2014)** used wheat bran, sea cabbage, inulin, ginger CO_2 extracts in liver pate formulation. The wheat bran was added from 10 to 20% instead of pork fat. The authors observed an increase in protein content and a decrease in fat as the quantity of plant components increased. As a result, a

prophylactic product with probiotic properties was obtained which could contribute to thyroid disease prevention.

Alexeeva et al. (2017) studied the possibility of applying vegetable food additives from wheat germ cake, alfalfa, and albumin seeds to pates from the liver. Their evaluation of the pate quality revealed that the addition of 15% vegetable food additive to the pate recipe had a positive effect on the water-binding, moisture-holding, and emulsifying ability as well as organoleptic characteristics. In terms of chemical composition, the protein content varied from 14.61 to 16.95 $g.100g^{-1}$, fat from 13.52 to 14.34 $g.100g^{-1}$ depending on the type of pate. The authors emphasize that the addition of vegetable food additives to the food dispersions does not require special technological methods and additional equipment.

Lukyanchenko and Makarova (2009) used germinated grains or lentil sprouts up to 15% to the mass of raw materials in the recipe of chicken liver-based meat pate. Adding to the pate high-protein legumes, such as lentils, allows us to obtain a product of high nutritional value. Water-binding capacity (WBC) characterizes the ability of a meat product to absorb and retain water **(Goh et al., 2012; Zhang et al., 2010)** and it significantly affects the rheological characteristics, organoleptic and sensory properties **(Rogov et al., 2009; Bakieva et al., 2019)**. The research results showed that meat pate with 10% of germinated wheat has the highest water-binding capacity (73%). This is probably because this sample contains 7% oat flour, which increases its strength and ability to retain moisture. Other samples contain less oat flour.

The degree and strength of water binding in minced meat also depends on the medium conditions (pH, temperature, salt composition) **(Damez and Clerjon, 2008; Rao et al., 1989)**. A similar trend in pH and WBC was observed in the work of **Veretnova and Safronova (2015)**, where combined minced meat with the addition of germinated wheat grain in the amount of 10% was investigated. The authors noted that the pH value of model minced meat coincides with the pH value of the control sample with the addition of up to 10% paste to the mass of minced meat instead of bread. The highest pH value was observed when 10% of germinated wheat grain was added. When more germinated wheat pasta (20 %) was added, the WBC value decreases by 1.5 %.

In the present study, the results of the organoleptic evaluation (Table 3) showed that the highest average score was obtained from the meat pate with a recipe that contains 10% of germinated wheat, and the lowest from the meat pate containing 20% of germinated wheat.

The biological value of proteins is determined by the optimal range of vital amino acids **(Alekseeva and Kolchina, 2019)**. The research results show that the meat pate with a content of 10% germinated wheat has a predominant content of essential and non-essential amino acids, as this sample contains a higher content of animal protein of meat trimmings. The amino acid composition showed a higher content of essential (7.742) and non-essential (10.612) amino acids in the pate sample with the addition of 10% GW. As the proportion of GW increases, these values decrease slightly. However, this amino acid composition is significantly higher in quantity than the amino acid in the pate control sample.

CONCLUSION

This study was focused on the assessment of the nutritional and biological value of meat pate enriched with germinated wheat. It revealed that in terms of chemical composition, meat pate with a content of 10% germinated wheat showed a high protein content of 14.36%. By analysing the amino acid composition, the pate enriched with 10% germinated wheat had an increased content of essential and non-essential amino acids compared to pates prepared with the addition of 15% and 20% of germinated wheat. The meat pate enriched with 10% germinated wheat provided the best amount of essential amino acids, had a denser consistency and maintained high consumer properties. This suggests that the nutritive value of meat pate enriched with germinated wheat is higher, demonstrating that wheat can be used in appropriate formulation to improve the organoleptic quality of meat pate.

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ASSESSMENT OF FLAVONOIDS AND PHENOLIC COMPOUND ACCUMULATION IN INVASIVE *SOLIDAGO CANADENSIS* L. IN SLOVAKIA

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ABSTRACT

Solidago canadensis L. was introduced to Europe as an ornamental plant from North America in 1645 and began to spread during the XIX-XX centuries. Nowadays the species is considered the most aggressive invasive species. On the other hand, *S. canadensis* is considered to be a medicinal plant. The raw material known as Herba Solidaginis includes herbs of *S. canadensis*, *S. gigantea*, and *S. virgaurea*. These species are known for their diuretic, anti-inflammatory, antimicrobial, antioxidant, antispasmodic properties. The purpose of our study was to analyze the chemical compounds and some biological properties of *S. canadensis*, growing in Slovakia, to evaluate its therapeutic potential. The total phenolic content (TPC) of the extracts from aerial parts of *S. canadensis* was determined by the Folin-Ciocalteu method. The evaluation of total flavonoid content (TFC) was performed by using a spectrophotometric method. The flavonoids content was expressed as rutin equivalents (mg REs) per g DW vegetal product. The phytochemical profile of *S. canadensis* extracts was assessed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The radical scavenging activity of samples was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Reducing power of extracts was determined by the phosphomolybdenum method. Total phenolic contents (TPC) and total flavonoid contents (TFC) of the extracts varied from 204.19 to 293.43 mg GAE.g^{-1} DW, and 64.99 – 175.25 g QE.g^{-1} DW, respectively; the best results were obtained for ethanol extract. Some phenolic compounds were identified by HPLC with significant amounts of rutin (211.20 µg.mL^{-1}), quercetin (122.08 µg.mL^{-1}), quercitrin (102.50 µg.mL^{-1}) and chlorogenic acid (147.00 µg.mL^{-1}). The DPPH values in the inflorescences were higher than in the leaves: the antioxidant activity of leaf extracts was in the range from 5.34 to 17.16 mg TE.g^{-1}, for inflorescences, this parameter ranged from 6.09 to 19.87 mg TE.g^{-1}. The high total phenolic compounds and flavonoids can be used as a valuable source of phytochemicals in herbal remedies. Our study of *S. canadensis*, growing in Slovakia, shows the promising potential that can be evaluated as an effective antioxidant and antimicrobial agent in herbal medicines.

Keywords: *Solidago canadensis*; flavonoid; phenolic compound; antiradical activity

INTRODUCTION

Solidago canadensis L. was introduced to Europe as ornamentals from North America in 1645 and began to spread during the XIX-XX centuries **(Lambdon et al., 2008; Vinogradova, Mayorov and Choroon, 2010)**. The specie is considered as the most aggressive invaders, which is defined by the European and Mediterranean Plant Protection Organization as invasive species having a high potential for spread and posing an important threat to the environment and biodiversity in the region **(Invasive Species Compendium, 2015)**. Abandoned and poorly managed agricultural areas contribute to the rapid spread and high density of goldenrod populations, and it was also recorded as one of the most common weeds in suburbs.

Invasive goldenrod is negatively evaluated because it reduces the abundance of native plants. On the other hand, goldenrod is considered to be medicinal plant. The raw material known as Herba Solidaginis includes herbs of *S. canadensis*, *S. gigantea,* and *S. virgaurea* **(Wichtl, 2013)**. Goldenrod has been traditionally used to treat inflammations of the urinary tract. Preparations from goldenrods have a well-defined diuretic, spasmolytic and hypotensive effect together with anti-inflammatory, bacteriostatic, and analgesic properties **(Apáti et al., 2003; Pawlaczyk et al., 2009; Deng et al., 2015)**. In addition to the above indications, preliminary studies of *Solidago* species have demonstrated that these plants contain a high-molecular-weight polysaccharide-protein complex that has strong cytotoxic activity against prostate cancer cells and an antitussive effect **(Ravichandiran and Deepa, 2012; Šutovská et al., 2013)**. There are also antitumor activities in the saponins fraction of *Solidago* species and antimicrobial, sedative, cytotoxic, and hypotensive effects in the essential oils of *Solidago* species **(Kołodziej, Kowalski and Kędzia, 2011; Vinogradova and Kuklina, 2018; Shelepova et al., 2018)**. The toxicity and contraindications for goldenrods preparations have not been reported, and the available information is based

mainly on studies conducted on the native European goldenrod (*S. virgaurea* L.).

The flavonoids and phenolic acids are one of the most numerous and widespread groups of natural constituents in the plant kingdom. They exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. Most research in recent years has been devoted to the antioxidant activity of flavonoids and phenolic acids, which is due to their ability to reduce the free radical formation and to scavenge free radicals. As a rule flavonoids and phenolic acids have low toxicity which, combined with high antioxidant capacity, makes these compounds extremely useful as pharmacological agents **(Pietta, 2000; Andersen and Markham, 2006)**. The effect of goldenrod preparations in urinary therapy is highly related to the biological action of flavonoids; they inhibit the enzyme neutral endopeptidase, which is responsible for the interaction of the atrial natriuretic peptide with the glomerulus, and, thus, they regulate the formation of urine via the excretion of sodium ions **(Melzig, 2004)**. However, despite the importance of flavonoids, investigations of these compounds in *Solidago* species are scarce. Studies on *S. canadensis* in China **(Wang et al., 2011; Deng et al., 2015)**, Lithuania **(Radusiene et al., 2015)** and Hungary **(Apati et al., 2002; Apáti et al., 2003; Apáti et al., 2006)**, *S. vigaurea* and *S. graminifolia* (L.) Elliot in Poland **(Roslon et al., 2014; Thiem et al., 2001)** and Romania **(Toiu et al., 2019)**, *S. caucasica* Kem.-Nath. and *S. dahurica* Kitag. in Russia **(Goryachkina, Buinov and Fedoseeva, 2012)** have been conducted.

Scientific hypothesis

Worldwide, works are being carried out the estimation of the content and accumulation of phenolic compounds during the growth of Solidago. The scientific hypothesis of the present study was to identify and quantify the concentrations of the principal phenolic compounds in widespread invasive *S. canadensis* in Slovakia to determine the importance of Solidago raw material as potential sources of phenolic compounds in foods and health-promoting ingredients for humans.

MATERIAL AND METHODOLOGY

Plant material

The population nearby Nitra city, Slovakia, has been observed. The population of *Solidago canadensis* (Figure 1) is very dense and occupies a large area. Thus, there is no shortage of this plant as a biological resource. Material has been dried in the shade, at a temperature of 20 – 30 °C. Because of our earlier studies proved the minimal concentration of functional ingredients in stems **(Shelepova et al., 2019)**, only leaves and inflorescences have been taken for analysis.

Figure 1 *Solidago canadensis* L.

Preparation of extracts

Air-dried plant material was mechanically ground with a laboratory mill to obtain a homogenous powder. All the samples of *S. canadensis* of approximately 0.1 g (weighed with 0.0001 g precision) were extracted in 10 mL of methanol (ME), ethanol (EE), acetone (AE) and aqueous (WE) extracts by ultra-sonication at 25 °C for 50 min. The prepared extracts were passed through a 0.22 μm filter and stored at 4 °C until analysis.

Quantitative analyses

The total phenolic content (TPC) of the extracts from *S. canadensis* aerial parts were determined by the Folin-Ciocalteu method **(Singleton, Orthofer and Lamuela-Raventós, 1999)**. The content in total phenolics was expressed as mg gallic acid equivalents (GAE).g^{-1} dry weight (DW) vegetal product. The experiments were performed in triplicate.

The evaluation of total flavonoid content (TFC) of the extracts from *S. canadensis* aerial parts was performed by using a spectrophotometric method **(Shafii et al., 2017)**. The flavonoids content was expressed as quercetin equivalents (mg QE).g^{-1} DW vegetal product.

HPLC conditions and analysis

The phytochemical profile of *S. canadensis* extracts was assessed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The equipment was an Agilent 1100 HPLC Series system (Agilent, Santa Clara, CA, USA) equipped with an autosampler, binary gradient pump, degasser, column thermostat (set at 48 °C), and UV detector. The mass spectrometer was an Agilent Ion Trap 1100 SL (LC/MSD Ion Trap VL, Agilent, Santa Clara, CA, USA) equipped with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The flow rate was 1 mL/min and the injection volume was 5 μL. The separation of compounds was performed on a reverse-phase analytical column (Zorbax SB-C18 100 × 3.0 mm i.d., 3.5 μm particle).

UV and MS modes were used for the detection of the compounds. A standard solution of polyphenols was used for collecting all spectra and integrating them into a library. The minimal concentration which produced a reproductive peak characterized by a signal-to-noise ratio greater than three was considered for calculation of the detection limits.

The retention times for the compounds were determined using reference standards and were based on the mass spectrum for each compound. Spiking samples with a solution containing each polyphenol (10 μg mL^{-1}) was used for accuracy check.

For identification of compounds, their retention times and the recorded ESI-MS spectra were compared with those of standards, which were obtained under identical working conditions. The method of the external standard was employed for the quantification of polyphenols in each extract and the calibration curves for a five-point plot were linear in the range 0.5 – 50.0 μg.mL^{-1} (R^2 >0.999).

Antioxidant activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to **Sánchéz-Moreno, Larrauri, and Saura-Calixto (1998)** with slight modification. The ethanol extract (1 mL) was mixed with 4 mL of DPPH solution (0.025 g of radical in 100 mL of ethanol). The absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) 10 –100 mg.L^{-1} (R^2 = 0.983) was used as a standard and the results were expressed in mg.g^{-1} Trolox equivalents (TE).

Reducing power of extracts

Reducing the power of extracts was determined by the phosphomolybdenum method of **Prieto, Pineda, and Aguilar (1999)** with slight modifications. The mixture of 1 mL of sample, 2.8 mL of monopotassium phosphate (0.1 M), 6 mL of sulfuric acid (1 M), 0.4 mL of ammonium heptamolybdate (0.1 M) and 0.8 mL of distilled water was incubated at 90 °C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Trolox 10 – 1000 mg.L^{-1} (R^2 = 0.998) was used as the standard and the results were expressed in mg.g^{-1} TE.

Chemicals

Acetonitrile was of HPLC grade and supplied by Sigma-Aldrich (Steinheim, Germany). Methanol, ethanol, and acetone were of analytical grade and were purchased from CentralChem (Slovakia).

Water was filtered through the Millipore HPLC grade water preparation cartridge (Millipore, Bedford, USA). The reference substances, chlorogenic acid (purity ≥95.33%), rutin trihydrate (purity 97.11%), and isoquercetin (purity ≥94.16%), were purchased from HWI ANALYTIK GmbH (Germany); quercitrin (purity ≥98.0%) was obtained from Sigma-Aldrich (Steinheim, Germany).

Statistical analysis

The statistically treated data are given as the arithmetical mean values and their standard errors. Data were submitted ANOVA and differences between means compared through the Tukey-Kramer test (p <0.05).

RESULTS AND DISCUSSION

Several *in vitro* and *in vivo* studies revealed that the presence of polyphenolic compounds in plant extracts could be related to important biological properties, such as antioxidant, immunomodulatory, antimicrobial, anticancer, prebiotic-like, vasodilating activities **(Brglez Mojzer et al., 2016)**.

Since flavonoids and phenolic acids are among the most important phytochemical constituents of some *Solidago* species, we evaluated their content in *S. canadensis* extracts. The results of the quantitative determination of total phenolics and flavonoids from different extracts (mg extract per g plant material) are presented in Figure 2.

Total phenolic contents (TPC) and total flavonoid contents (TFC) of the extracts were determined; the TPC

and TFC of the aerial part extracts varied from 204.19 to 293.43 mg GAE.g^{-1} DW, and 64.99 – 175.25 g QE.g^{-1} DW, respectively. The highest values were observed in the aerial parts of *S. canadensis* ethanol extract, with total phenolics (293.42 mg GAE.g^{-1} extract) and flavonoids (175.25 mg QE.g^{-1} extract). In the aerial parts, the amount of TPC under investigation can be arranged in descending order: EE > ME ≈ AE ≈ WE (p <0.05). The EE (175.25 QE.g^{-1}) extracts showed the highest TFC, for ME (141.05 QE.g^{-1}) and AE (144.48 QE.g^{-1}) were found to be similar, the lowest level was in the aqueous extracts (WE) (64.99 QE.g^{-1}).

Obtained results were in good agreement with the ones presented by **Deng et al. (2015)**, who determined the total polyphenols and flavonoid contents in ethanol extracts from leaves of *S. canadensis*. Similarly, the highest contents of bioactive compounds were found in ethanol extracts from *S. graminifolia* (TPC – 192.69 mg.g^{-1}, TFC – 151.25 mg.g^{-1}) **(Toiu et al., 2019)**, *S. microglossa* (TPC – 226.0 mg.g^{-1}, TFC – 115.2 mg.g^{-1}) **(Sabir et. al., 2012)** and *S. virgaurea*, *S. canadensis*, *S. gigantea* (TFC – 118.0; 156.0 and 156.0 mg.g^{-1}, respectively) **(Kołodziej Kowalski and Kędzia, 2011)**. Recent research carried out by **Woźniak et al. (2018)** on *Solidago* sp. showed that the methanol extracts from aerial parts from *S. canadensis* contain 82.1 mg flavonoids per g extract and 130.5 mg polyphenols per g extract, which is lower than the content determined in *S. canadensis* in our study. Studies pointed out that certain factors, such as the extraction method, the type of solvents and solubility of active compounds, the temperature, the extraction time, and the ratio of solvent-to-sample are essential parameters that greatly influence the yield and quality of obtained extracts from plant materials.

Also, it should be noted that the flavonoid content meets the quality criteria of European Pharmacopoeia mentioned at *Solidaginis herba* (minimum 2.5% flavonoids).

Taking into account that the highest amounts of total flavonoids and total phenolics were observed in *S. canadensis* extracts, the HPLC analysis for identification and quantification of polyphenolic compounds was carried out and the obtained results are presented in Table 1.

Three groups of phenolic compounds have been identified in the aerial parts of *S. canadensis* – phenol carboxylic acids, such as chlorogenic acid, caftaric acid and t-ferulic acid (and other acids according to the mass spectrum), and flavonoids, such as flavonol glycosides (rutin, hesperidin, quercitrin), flavonol aglycones (quercetin, kaempferol), and catechin (Table 1). These compounds had chromatographic and spectral characteristics similar to those previously identified in *S. canadensis* L. **(Apáti et al., 2003; Apáti et al., 2006; Woźniak et al., 2018)**.

Rutin (200.45 – 211.20 mg.g^{-1}), quercetin (121.74 – 122.41 mg.g^{-1}), quercitrin (102.50 – 125.70 mg.g^{-1}) and chlorogenic acid (134.95 – 147.00 mg.g^{-1}) were the major active compounds in *S. canadensis* aerial parts ME, EE and AE extracts. The WE extract was rich in polar compounds, especially chlorogenic acid (834.5 mg.g^{-1}) and vanillic acid (154.08 mg.g^{-1}), which was the major phenolic acid present, and represented 1186.87 mg.g^{-1} of the extract.

The majority of flavonoids in the WE extract were identified as monoglycosides (127.67 mg.g^{-1}) of kaempferol and quercetin (Table 1). Also, other compounds were detected that corresponded to rutin (44.77 mg.g^{-1}), quercitrin (35.84 mg.g^{-1}) and hesperidin (11.55 mg.g^{-1}).

Rutin (quercetin-3-rhamnosyl glucoside) and quercetin were the main flavonoids identified in *S. canadensis* aerial parts extracts. These compounds are the important contributors to the antioxidant, anti-inflammatory, vasodilator, antiatherosclerotic, antihypercholesterolemic, anti-obesity, and angioprotective potential of plant extracts **(Yang, Guo and Yuan, 2008; D'Andrea, 2015)**. Chlorogenic acid (5-O-caffeoylquinic acid) is a plant secondary metabolite widely distributed in coffee, tea, many fruits, vegetables, and herbs. Recent studies on this phenolic compound have demonstrated multiple biological properties, such as antioxidant, anti-inflammatory, antibacterial, antiviral, cardioprotective, hepatoprotective, neuroprotective, antihypertensive, anti-obesity, antidiabetic, antiapoptotic. Additionally, many *in vivo* studies and clinical trials have been done concerning the health benefits of chlorogenic acid as a nutraceutical agent for the prevention and treatment of metabolic syndrome and associated diseases **(Naveed et al., 2018)**.

Due to their multiple biological activities, the phenolic compounds from some *Solidago* species have been formerly examined. *S. gigantea* methanol extracts contain the chlorogenic acid (0.1 mg.g^{-1} DW plant material) and the quercitrin (4.5 mg.g^{-1} DW plant material) **(Woźniak et al., 2018)**, ethanol extracts of *S graminifolia* contain the chlorogenic acid (997.88 mg.100g^{-1} extract), quercitrin (431.59 mg.100g^{-1}) and hyperoside (253.19 mg.100g^{-1}) **(Toiu et. al., 2019)**. Ethanolic extract of leaves of *S. microglossa* contains the quercetrin (51.9 mg.g^{-1}), gallic acid (24.1 mg.g^{-1}), rutin (3.82 mg.g^{-1}), and quercetin (2.57 mg.g^{-1}) **(Sabir et al., 2012)**. The hydroalcoholic extract of *S. canadensis* found rutin (8.93% w/w) as the main compound, as well as caffeic acid derivatives **(Apáti et al., 2006)**.

Because the most significant differences of *S. canadensis* in different extracts were determined by rutin, quercetin, quercitrin, and chlorogenic acids establishing the metabolic profile of polyphenols that may contribute to ensuring plants material of high quality and safety for more efficient phytopharmaceuticals.

Considering that plant extracts are complex mixtures of numerous natural compounds with synergic or additive effects, the investigation on new sources of chlorogenic acid could reveal possible applications in medicine and pharmacy.

Plants are a potential source of natural antioxidants, which act as reducing agents, hydrogen donators, oxidants, and free radical scavengers. The antioxidant activity evaluation was carried out by DPPH and RP radical scavenging activity assays. These frequently used methods are rapid and valuable for the evaluation and quantification of the free radical scavenging activity of natural compounds from plant extracts. As shown in Table 2, there were wide variations in the DPPH and RP of the leaves and inflorescences *S. canadensis*, ranging from 5.34 to 19.87 mg TE.g^{-1}, and from 66.78 to 258.22 mg TE.g^{-1}, respectively.

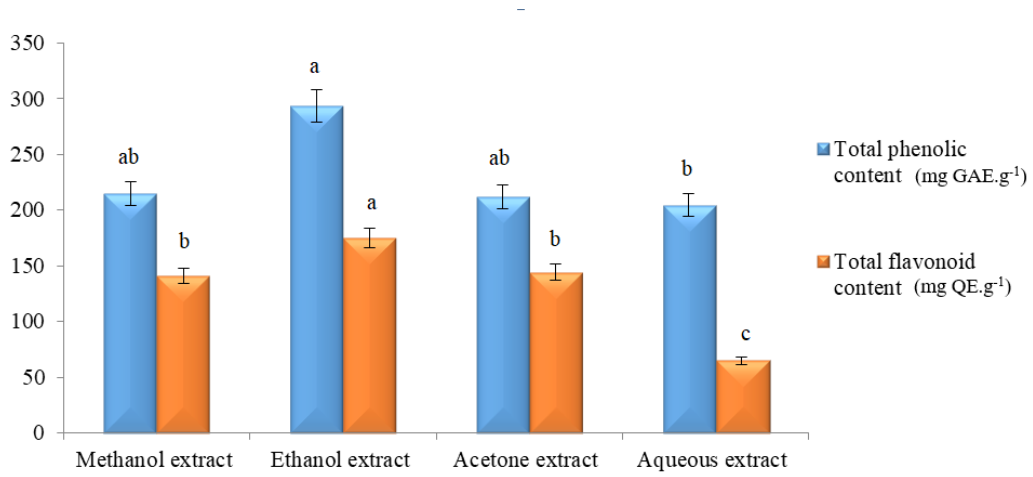

Figure 2 Total phenolic and flavonoid contents (mg.g^{-1} extract) in *Solidago canadensis* extracts (means in columns followed by different letters are different at p <0.05; each value represents the mean of three independent experiments (±SD).

Table 1 Content of phenolics and flavonoids (mg.g^{-1} extract) in different extracts of aerial parts of *Solidago canadensis*; LOQ variation for antioxidant compounds.

Compound	LOQ, µg.mL^{-1}	Methanol extract	Ethanol extract	Acetone extract	Water extract
Gallic acid	<0.1	3.02 ±0.41	<0.1	<0.1	28.05 ±1.04
Protocatechuic acid	<0.1	19.50 ±0.74	26.33 ±0.59	43.50 ±1.86	71.84 ±2.05
Chlorgenic acid	<0.1	146.05 ±5.05	147.00 ±4.85	134.95 ±4.50	834.50 ±9.75
Epicatechin	<0.1	45.50 ±2.05	32.00 ±1.95	20.15 ±1.78	11.99 ±0.95
Catechin	<0.1	12.01 ±0.78	18.04 ±0.82	<0.1	<0.1
Caffeic acid	<0.2	<0.2	<0.2	65.95 ±2.05	<0.2
Vanillic acid	<0.5	<0.5	<0.5	54.49 ±2.09	154.08 ±5.11
Syringic acid	<0.1	26.45 ±1.01	<0.1	78.09 ±2.85	<0.1
Coumaric acid	<0.1	17.99 ±0.74	19.00 ±1.05	23.50 ±1.25	72.30 ±2.18
t-Ferrulic acid	<0.2	55.02 ±1.98	48.25 ±1.08	32.85 ±1.84	25.38 ±0.75
Hydroxy flavanon	<0.2	25.41 ±0.55	37.60 ±0.99	27.84 ±0.65	<0.2
Rutin	<0.1	200.45 ±5.95	211.20 ±6.50	211.14 ±5.80	44.77 ±0.67
Quercitrin	<0.2	112.85 ±4.05	102.50 ±3.95	125.70 ±4.20	35.84 ±0.55
Quercetin	<0.2	121.74 ±4.10	122.08 ±5.05	122.41 ±4.80	68.95 ±0.93
Hesperidin	<0.1	97.65 ±2.07	51.87 ±1.85	58.45 ±1.37	11.55 ±0.28
Kaemferol	<0.2	<0.2	45.00 ±1.24	28.05 ±0.95	58.72 ±1.05

Note: LOQ: limit of quantification. Values are expressed as the mean ±SD (n = 3).

Table 2 Free radical scavenging activity by DPPH and RP assays of *Solidago canadensis* L. extracts.

Extracts	DPPH (mg TE.g^{-1})		RP (mg TE.g^{-1})	
	leaves	inflorescences	leaves	inflorescences
Methanol extract	15.12 ±0.47^{ab}	16.27 ±0.51^{b}	190.05 ±2.18^{b}	225.80 ±3.07^{a}
Ethanol extract	10.30 ±0.35^{b}	12.14 ±0.40^{c}	128.95 ±2.75^{c}	124.74 ±2.81^{b}
Acetone extract	17.16 ±0.53^{a}	19.87 ±0.42^{a}	214.70 ±3.01^{a}	228.22 ±2.54^{a}
Aqueous extract	5.34 ±0.28^{c}	6.09 ±0.21^{d}	66.78 ±1.51^{d}	97.15 ±1.75^{c}

Note: Values are expressed as the mean ±SD (n = 3). Means in columns followed by different letters are different at p <0.05.

The DPPH radical is a stable lipophilic free radical and is a measure of non-enzymatic antioxidant activity of plant extracts **(Deng et al., 2015)**. The higher the DPPH values, the higher the antioxidant activity. The DPPH values in the inflorescences were higher than in the leaves under all kinds of extraction. We found that the antioxidant activity of investigated extracts of leaves *S. canadensis* was in the range from 5.34 to 17.16 mg TE.g^{-1} (Table 2). For inflorescence extracts of *S. canadensis,* this parameter ranged from 6.09 to 19.87 mg TE.g^{-1}. The AE extracts of inflorescences and leaves had the highest DPPH radical scavenging activity, followed by the ME extracts; the poorest antioxidant activity was found in the WE extracts. The scavenging effect on the DPPH radical in the leaves and inflorescence extracts decreased in the order: AE >ME >EE >WE.

The RP assay is based on the redox reaction of the reduction of Mo (VI) to Mo (V) ion in the presence of a reducer and is expressed as absorbance value at 700 nm, in which a greater absorbance corresponded to a higher reducing activity. All kind's extracts of inflorescences of *S. canadensis* exhibited more significant antioxidant activity compared with the leaves extracts (p <0.05). The AE inflorescence extracts had the highest reducing power, followed by the ME extracts; the WE extracts exhibited the smallest RP. The RP ascended in the order: EE <WE <ME ≈ AE for the inflorescences extracts and WE <EE <ME <AE for the leaf extracts.

The results of our research indicate that the antioxidant potential may be due to the presence of phenolic acids and flavonoids, which were found in significant amounts in *S. canadensis* all kinds of extracts. Each phenolic compound reacts differently and its antioxidant effect is closely related to the structure, for example, rutin and quercetin contain vicinyl dihydroxyl groups. The presence of vicinyl dihydroxyl groups was shown to affect the ability of phenols to inhibit iron and copper-catalyzed production of initiating radical species **(Yang, Guo and Yuan, 2008)**. Thus, it is likely that metal chelation and/or free radical scavenging properties contribute to the inhibition of glucose autoxidation by rutin and quercetin metabolites containing vicinyl dihydroxyl groups. While chlorogenic acid inhibits lipid oxidation in oil-in-water emulsion through a complex effect, metal-chelating in the hydrophilic phase and free radical scavenging in the hydrophobic phase **(Santana-Gálvez, Cisneros-Zevallos and Jacobo-Velázquez, 2017)**.

CONCLUSION

S. canadensis in Slovakia was a poorly studied species of the genus *Solidago*, which includes medicinal plants known for their diuretic, anti-inflammatory, antimicrobial, antioxidant, antispasmodic properties. Saponins, flavonoids, salicylic acid derivatives, tannins, etc. contained in the plant material are active substances of medicinal products. We have studied the content of phenolic compounds in various extracts and the best results were obtained for ethanol extract. Some phenolic compounds were identified by HPLC in methanol, ethanol, acetone, and aqueous extracts of the aerial parts of *S. canadensis* with significant amounts of rutin, quercetin, quercitrin, and chlorogenic acid. The high total phenolic compounds and flavonoids content can be used as a valuable source of phytochemicals in herbal remedies. Our study of *Solidago canadensis*, growing in Slovakia, shows the promising potential that can be evaluated as an effective antioxidant and antimicrobial agent in effective herbal medicines.

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ADAPTATION OF TWO-DIMENSIONAL ELECTROPHORESIS FOR MUSCLE TISSUE ANALYSIS

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ABSTRACT
It is important to understand the molecular mechanisms that take place in muscle tissues and to predict meat quality characteristics. One of the most popular methods is two-dimensional electrophoresis, which allows us to visualize, share and identify different molecules, including meat proteins. However, the standard conditions of this method are not universal for all types of raw material, so the authors suggest a new variation of two-dimensional electrophoresis for muscle tissue analysis. Samples were tested by the classical version of isoelectric focusing (cathode buffer in the top and anode buffer in the bottom chamber of the electrophoresis cell) and its variation (anode buffer in the top and cathode buffer in the bottom chamber of the electrophoresis cell). Next, extruded gels were incubated in two different buffer systems: the first was equilibration buffer I (6 M urea, 20% w/v glycerol, 2% w/v SDS and 1% w/v Ditiothreitol in 375 mM Tris-HCl buffer, pH 8.8) followed by equilibration buffer II (6 M urea, 20% w/v glycerol, 2% w/v SDS and 4% w/v iodoacetamide in 375 mM Tris-HCl buffer pH 8.8 and the second, buffer A, consisting of 5 M urea, 2% w/v SDS, 5% v/v mercaptoethanol, 62.5 mM Tris-HCl buffer, pH 6.8 and 0.01% w/v bromophenol blue. Electrophoretic studies of muscle tissue revealed the best protein separation after changing the direction of the current (authors' variation), while no differences were detected after changing incubation buffers.

Keywords: two-dimensional electrophoresis; muscle protein; isoelectric focusing; meat

INTRODUCTION
Meat and meat products will always play an important role in the human diet as a source of high-grade protein. There is a wide variety of meat processing products, the range of which is regularly updated. Depending on the processing technology, meat proteins undergo extensive modifications that affect their quality, shelf life, nutritional properties, and health effects. At the same time, tenderness and juiciness are the criteria most judged by consumers **(Cao et al., 2020; Hollung et al., 2007)**.

These criteria are influenced by the following factors: genetics, environment, animal welfare, and further processing. The molecular mechanisms underlying such processes are still of interest. However, genetic information remains static throughout the life of the body, while the protein composition is dynamic and changes depending on factors affecting protein synthesis or degradation **(Peng and Gygi, 2001)**. Thus, proteomics analysis makes it possible to better understand the molecular mechanisms that occur in tissues and to predict the most important characteristics, particularly in the case of meat **(Bendixen, 2005; Bendixen et al., 2005; Zamaratskaia and Li, 2017)**.

Proteomic methods over the past decade have found increasing application in various fields of science and agriculture. To date, proteomic tools have improved significantly, and mass spectrometric methods are being developed very rapidly. Most proteomic methods are based on the separation of proteins in at least two directions, using chromatographic or electrophoretic methods, with further identification using mass spectrometry methods **(Soares et al., 2012; Suman et al., 2014)**. In this paper, attention is focused on the method of two-dimensional gel electrophoresis (2-DE), because it is still an excellent way to visualize protein components and widely used in meat science, with an emphasis on sample preparation and aspects of isoelectric focusing (IEF).

Traditionally, 2-DE is carried out by fractionation of proteins according to two different physicochemical parameters. In the first direction, proteins are separated by charge according to their isoelectric point with IEF, and then in the second direction by their molecular weight with polyacrylamide gel electrophoresis (PAAG). Some variations of conducting 2-DE are discussed below to identify the most optimal variant for analysis of the meat proteome.

Scientific hypothesis

The classical variation of two-dimensional electrophoresis does not fully reveal muscle tissue proteins. It is necessary to select optimal conditions for the analysis. Changing the IEF parameters and additional incubation in lysis buffers will increase the resolution of the 2-DE method for muscle proteins.

MATERIAL AND METHODOLOGY

Materials

Chemical reagent: Urea, Thiourea, Ditiothreitol, Sodium hydroxide (NaOH), Glycerol, Sodium dodecyl sulfate (SDS), Tris, Acrylamide, Ammonium persulfate (APS), 2-Propanol, Acetic acid (PanReac, Spain); Bis-acrylamide, Tetramethylethylenediamine (TEMED), Mercaptoethanol, Bromophenol blue, Glycine, Coomassie Brilliant Blue G-250, Triton X-100 (Helicon Russia); Ampholyte (Serva, Germany) and Phosphoric (V) acid (H_3PO_4) (Component-reaktiv, Russia).

The object of the study was the Vietnamese Pot-bellied from healthy females of 60 – 65 days old pig *L. dorsi* muscle. Samples were taken within 20 minutes after slaughter and placed in dry ice. Frozen muscle tissues (50 mg) were homogenized in 1 mL 7 M Urea, 2 M Thiourea, 1% Ditiothreitol, 0.4% Triton X-100, 2% pH 3-10 Ampholyte. Homogenates were centrifugated with an acceleration of 20 000 g for 20 minutes. Three samples, obtained from different animals, were studied by two-dimensional electrophoresis in two variations of isoelectric focusing (IEF) and two methods of gel incubation after IEF. Figure 1 shows the experimental design.

Two-dimensional gel electrophoresis (2-DE)

IEF in the first dimension was performed at 3650 $V.h^{-1}$. The anodic and cathodic electrode solutions used for IEF were 0.01 M Phosphoric (V) acid and 0.02 M Sodium hydroxide, respectively, in 2.4 mm × 160 mm tube gels.

In the first classical version of IEF, the cathode buffer (0.02 M sodium hydroxide) was in the upper chamber of the electrophoresis cell, and the anode buffer (0.01 M orthophosphoric acid) was in the lower one. In the second variation of IEF, the electric current direction was changed: the anode buffer was in the upper chamber, and the cathode buffer was in the lower one.

After the IEF, gels were incubated in two different ways: extruded tube gels were incubated for 10 min, in 2.5 mL of equilibration buffer I (6 M urea, 20% w/v glycerol, 2% w/v SDS and 1% w/v Ditiothreitol in 375 mM Tris-HCl buffer, pH 8.8), followed by equilibration buffer II (6 M urea, 20% w/v glycerol, 2% w/v SDS and 4% w/v iodoacetamide in 375 mM Tris-HCl buffer, pH 8.8); extruded tube gels were incubated for 10 – 15 min, in 2.5 mL buffer A (5 M urea, 2% w/v SDS, 5% v/v mercaptoethanol, 62.5 mM Tris-HCl buffer, pH 6.8 and 0.01% w/v Bromophenol blue).

For SDS-PAGE (12% T, 2.6% C) equilibrated tube gels were transferred to a 12.5% polyacrylamide gel (170 mm × 180 mm × 1.5 mm). Electrophoresis was carried out with a gel running buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% w/v SDS at 30 mA per gel until the bromophenol blue front had reached the lower edge of the gel. Experimental molecular weights were detected relatively to marker proteins – 250; 150; 100; 70; 50; 40; 30; 20; 15; 10; 5 kDa (Thermo Scientific, Lithuania).

Protein visualization and image analysis

Protein spots were visualized staining by solution Coomassie (0.05% w/v Coomassie Brilliant Blue G-250, 10% v/v Acetic acid and 25% v/v 2-Propanol) for 1 hour. The destaining procedure was carried out by incubating in 10% v/v Acetic acid for 15 minutes several times until the background of the gel became transparent.

For computerized densitometry, two-dimensional electropherograms were used in a wet state. Their full digital images and/or images of individual fragments were obtained using a Bio-5000 plus scanner (Serva, Germany). Scanned images were analyzed with ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland). Spots were detected and quantified automatically with minimum thresholds: saliency – 11, min area – 5 and smooth – 3. The relative optical density (OD) and relative volume were computed to correct for differences in gel staining. These measures take into account variations due to protein loading and staining, by considering the total OD or volume over all the spots in the gel. The digitized 2DE images of the cortex were then compared by the matching method **(Grove et al., 2006)**.

Protein spots on muscle tissue two-dimensional electropherograms were interpreted following the Swiss-Prot database **(O'Donovan et al., 2002)** and the Muscle organ proteomics database **(Kovaleva et al., 2013)**.

Statistical analysis

The experimental data were analyzed using ordinary one-way ANOVA (between gels, obtained with different variation IEF) by ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland).

A p value <0.05 was considered to indicate a significant difference. All results are presented as mean ± SD from at least three independent experiments.

RESULTS AND DISCUSSION

In most scientific works, IEF is performed using a thin layer of gel deposited on a plastic substrate (IPG Dry Strip) to speed up and simplify the process. **(Naveena et al., 2017; Di Luca et al., 2016; Lee, Saraygord-Afshari and Low, 2020)**. However, the glass-tube IEF, despite process complexity, has an advantage in the resolution and protein loading volume in the gel **(Matsumoto et al., 2019)**. Based on foregoing, for this experiment, we selected glass-tube IEF.

In accordance with the experimental design, depicted in Figure 1, four 2-DE variations were performed; the obtained electropherograms are presented in Figure 2.

A different distribution of pig muscle protein was noted for molecular weights (MW) and isoelectric points (pI).

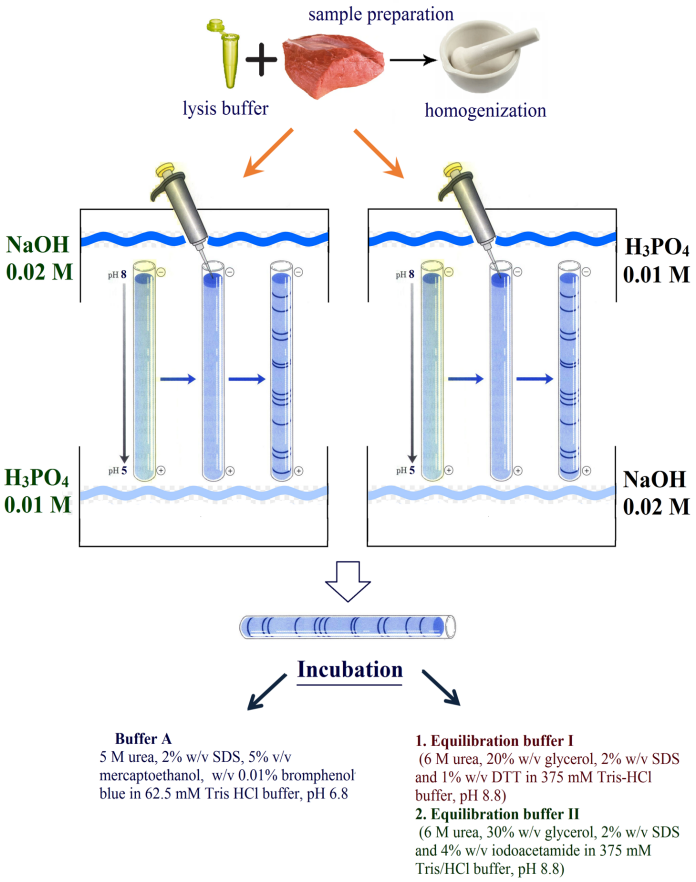

Figure 1 Experimental design of pig *L. dorsi* 2-DE.

Table 1 Results of the densitometry analysis.

	Name of gel			
№ Spot	**A (vol ± SD)**	**B (vol ± SD)**	**C (vol ± SD)**	**D (vol ± SD)**
1	8.19 ±1.1622 x 10^7	5.75 ±0.90 x 10^7	7.47 ±0.06 x 10^7	7.34 ±0.10 x 10^7
2	10.18 ±0.2622 x 10^7	10.70 ±0.14 x 10^7	11.48 ±0.26 x 10^7	12.00 ±0.34 x 10^7
3	9.37 ±0.2022 x 10^7	8.97 ±0.44 x 10^7	8.58 ±0.53 x 10^7	9.64 ±0.63 x 10^7
4	4.39 ±0.2522 x 10^7	9.47 ±.1.05 x 10^7	7.51 ±0.15 x 10^7	7.20 ±0.23 x 10^7
5	1.92 ±0.39 x 10^7	2.71 ±0.72 x 10^7	2.95 ±0.54 x 10^7	4.03 ±0.66 x 10^7
6	10.65 ±0.62 x 10^7	9.42 ±0.84 x 10^7	14.42 ±0.60 x 10^7	13.23 ±0.98 x 10^7
7	8.19 ±0.41 x 10^7	5.75 ±1.22 x 10^7	7.47 ±0.07 x 10^7	7.34 ±0.64 x 10^7
8*	0.64 ±0.16 x 10^7	1.04 ±0.88 x 10^7	13.30 ±0.50 x 10^7	11.82 ±0.97 x 10^7
9*	0.34 ±0.05 x 10^7	0.42 ±0.14 x 10^7	6.35 ±0.60 x 10^7	6.23 ±0.74 x 10^7
10*	2.42 ±0.61 x 10^7	1.26 ±0.58 x 10^7	5.84 ±0.64 x 10^7	4.63 ±0.43 x 10^7
11*	1.24 ±0.02 x 10^7	1.57 ±0.27 x 10^7	10.68 ±0.74 x 10^7	9.68 ±0.85 x 10^7
12*	0.40 ±0.25 x 10^7	0.89 ±0.31 x 10^7	13.37 ±1.20 x 10^7	10.78 ±0.92 x 10^7
13*	0.29 ±0.08 x 10^7	0.45 ±0.11 x 10^7	4.91 ±0.27 x 10^7	5.45 ±0.59 x 10^7
14*	0.24 ±0.04 x 10^7	0.13 ±0.02 x 10^7	3.16 ±0.54 x 10^7	3.19 ±0.71 x 10^7
15*	4.23 ±0.25 x 10^7	0.24 ±0.08 x 10^7	5.61 ±0.44 x 10^7	2.47 ±0.37 x 10^7

Note: Spot Vol** were normalized by total valid spot volume and mean of value from duplicate analytical gels from three replicates. Data represented are means ±SD of three independent experiments. *Significant differences were found between IEF variations in the distribution of values between gels within the same sample with p <0.05. **Vol: The volume of a spot is the sum of the background-subtracted gray values of all pixels delimited by the spot border. By default, the background is defined as the minimum gray value on the spot border.

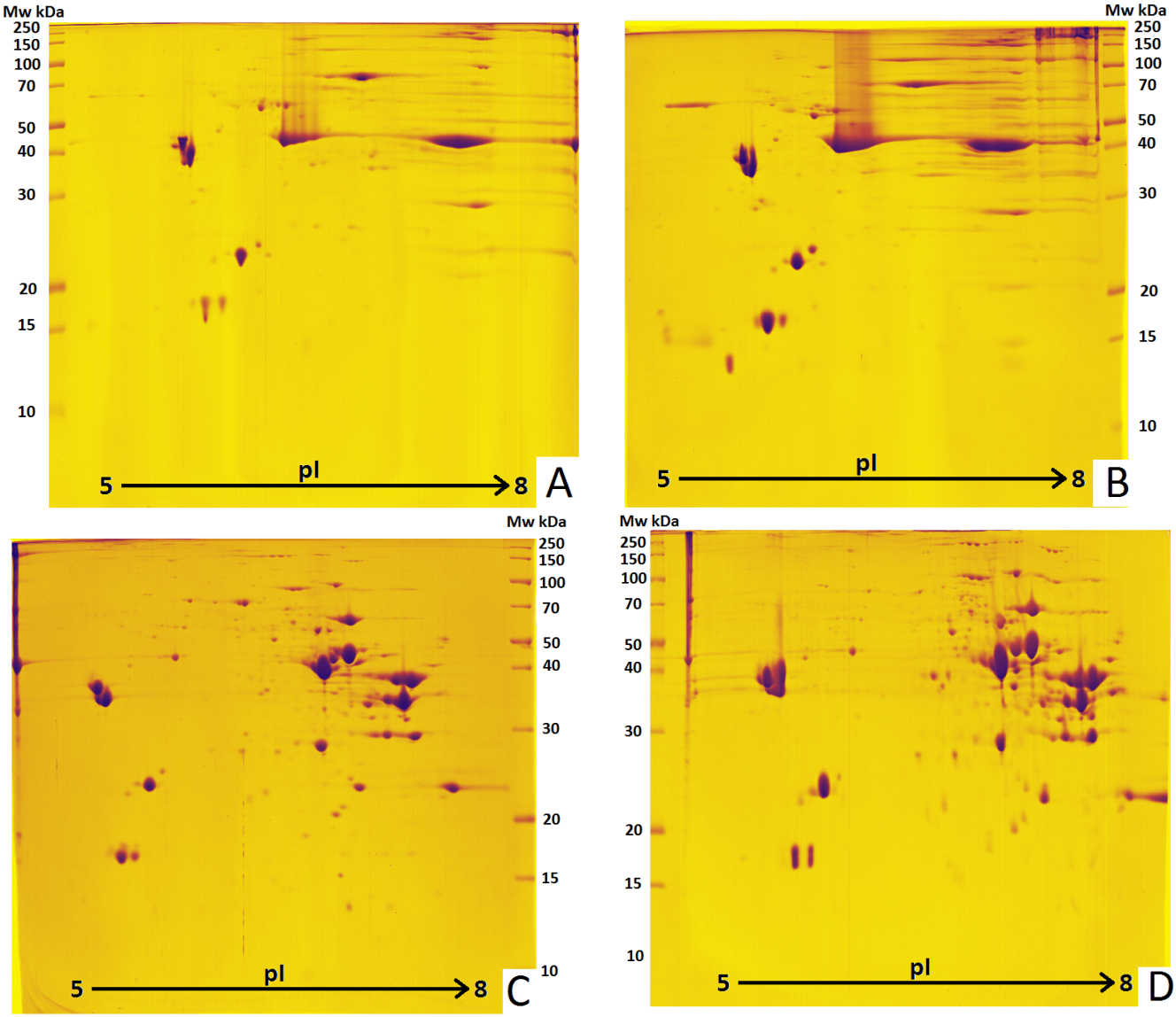

Figure 2 2-DE of pig *L. dorsi*. Note: A – 0.02 M NaOH were from above, 0.01 M H_3PO_4 from below, tube gels were incubated in equilibration buffer I. and II; B – 0.02 M NaOH were from above, 0.01 M H_3PO_4 from below, tube gels were incubated in buffer A; C – 0.01 M H_3PO_4 were from above, 0.02 M NaOH were from below, tube gels were incubated in equilibration buffer I and II; D – 0.01 M H3PO4 were from above, 0.02 M NaOH were from below, tube gels were incubated in buffer A.

In the classical version of IEF **(O'Farrell, 1975; Hirano, 1982; Kimura et al., 2003)**, when 0.02 M NaOH is in the upper chamber of the electrophoretic cell (Figure 2A), good protein separation is observed within a pI range of 5 to 6.5, such as tropomyosin beta chain (Mw 33.5 kDa, pI 4.80) **(D´Alessandro et al., 2011; Peng et al., 2013)**, tropomyosin alpha-3 chain (Mw 33.5 kDa, pI 4.71) **(Davoli et al., 2000)**, myosin light chain 3-like (Mw 22.0 kDa, pI 5.24) and myosin light chain 1/31 (Mw 21.0 kDa, pI 5.80) **(Montowska and Pospiech, 2012; Kovaleva et al., 2013)**.

Moreover, on two-dimensional electropherograms with incubation in buffer A (Figure 2B), these proteins are most clearly identified **(Ros et al., 2002; Montowska and Pospiech, 2007; Paredi, Mori and Mozzarelli, 2018)**. At the same time, in the alkaline zone of the gel, a blurred image of protein fractions is observed. A completely different gel was obtained when the current direction changed during the IEF, when 0.01 M orthophosphoric acid was in the upper chamber of the electrophoretic cell. Clear and well-defined protein spots were obtained with uniform distribution over the entire gel area **(Chernukha et al., 2017)**. Presumably, this was because proteins with acidic and neutral pI are more highly represented in muscle tissue, resulting in a higher proportion of positively charged proteins. Since the current always goes in the direction from "plus" to "minus", when changing the direction of the current flow, it is easier for positively charged proteins, which are always layered on top to move to the lower chamber of the electrophoretic cell, where a negative charge (cathode) is created **(Colangeli et al., 2018)**.

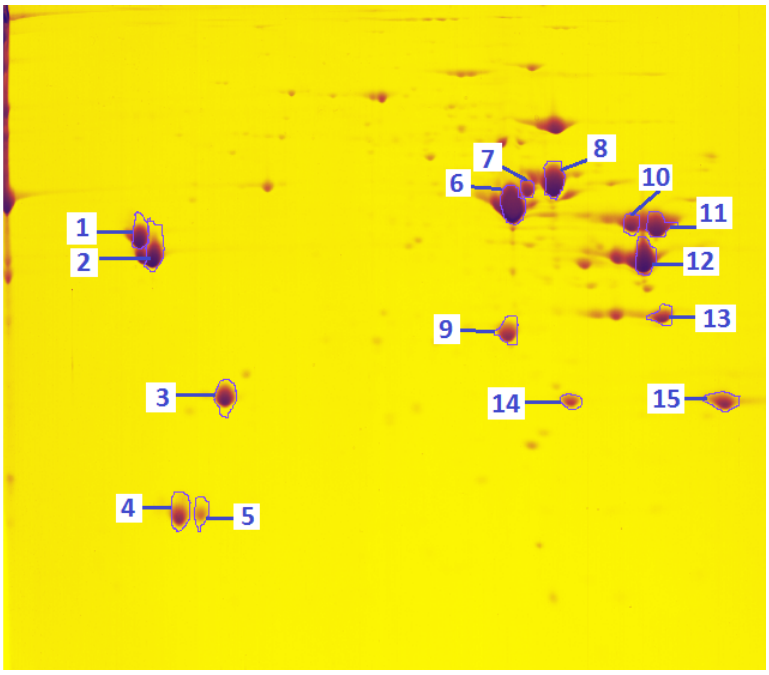

Figure 3 2-DE of pig *L. dorsi* with the designation of structural muscle proteins for densitometric assessment.

Interestingly, during incubation in equilibration buffer I and II (Figure 2C), protein spots with a molecular weight greater than 50 kDa were better visualized **(Vasilevskaya and Akhremko, 2019)**. In contrast, when incubated in buffer A (Figure 2D), protein spots with a molecular mass of less than 40 kDa were better visualized. The latter variation was used in a study by Kovalev **(Kovalyov et al., 2006; Zvereva et al., 2015)**, and analysis of the results found that this method, as in the case of incubation in equilibration buffers, allows the best detection of muscle tissue proteins.

Major structural proteins of pig muscle tissue (Figure 3) were found **(Montowska and Pospiech, 2012)** and subjected to densitometric analysis (Table 1). The Fold change index **(Persike et al., 2018**) was calculated. Fold change index is the ratio between the volume of protein spots with the highest average value and the lowest average value. Eight fractions were detected (≥2-fold change, p <0.05) with increased spot volume by at least 2 times compared with the classical version of the IEF (marked * in Table 1).

Thus, the variation of the IEF, when sodium hydroxide is in the upper chamber of the electrophoresis cell, has a lower resolution and does not properly detect protein fractions in the alkaline zone. So, in Figures 2A and 2B, proteins in the right half of the gel are noticeably out of focus. It is necessary to increase the number of volt-hours by at least 40% (up to 7 – 8 hours) for better protein separation in this area.

After changing the direction of electric current during the IEF, the process takes 4 hours. As a result, at least two times more protein spots are detected on 2-DE, including major structural muscle proteins, such as glyceraldehyde-3-phosphate dehydrogenase **(Han et al., 2019)**, troponins group **(Mora et al., 2016; Drousiotis et al., 2020)**, 3-hydroxyacyl-Coenzyme A dehydrogenase, beta-enolase and others **(Nolan et al., 2019)**.

CONCLUSION

The results of electrophoretic studies showed that the best option for the separation of meat proteins is to change the direction of the current. Thus, when the anode buffer (0.01 M orthophosphoric acid) is in the upper chamber of the electrophoretic cell, and the cathode buffer (0.02 M sodium hydroxide) is in the lower one, the most informative picture is obtained. Incubation in both buffer A and equilibration buffers can also be used.

Densitometric analysis showed that the use of new parameters allows us to identify a larger number of proteins (almost 2 times). An increase in the color intensity of certain fractions is also noted. Thus, the proposed variation of the IEF can be used as the main one for muscle proteins electrophoretic analysis, since it requires less time and has a higher resolution.

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EVALUATION OF STORAGE METHODS OF BEEF BY MICROBIOLOGICAL AND CHEMICAL INDICATORS

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ABSTRACT
Meat and meat products are a major part of a person's ration. However, due to their high nutritional value, they are a favorable environment for the development of microorganisms and require refrigerated storage. The purpose of this work was to evaluate the storage methods for refrigerated and frozen beef by microbiological and chemical parameters and to suggest criteria for evaluating beef by the content of psychrotrophic microorganisms. It was found out that the storage of beef meat with an initial mesophilic bacterial content of about 4.88 log CFU.cm^{-2} of surface and psychrotrophic bacteria 3.79 log CFU.cm^{-2} at temperature 0 °C is only possible for 8 days, further, the microbiological indices exceed the acceptable standards. Investigation of the dynamics of microflora reproduction during the storage of beef in the frozen state at temperature -2 to -3 °C for 20 days established a decrease in 1.3 times the number of mesophilic bacteria in 10 days of storage. At the same time, the number of psychrotrophic microorganisms during this storage time was increased in 4.5 times, and 20 days in 7.9 times and amounted to 5.3 log CFU.cm^{-2} of surface area. This indicates that the storage of meat in the frozen state inhibits or completely stops the development of mesophilic microorganisms for 20 days. It was found out that storing of beef in the cooled state at a temperature of 0 ±0.5 °C for more than eight days is impractical, as its biochemical indices are worsening and signs of spoilage are appearing. At the same time, storing of beef in the frozen state at a temperature of -2 to -3 °C for 20 days does not cause such significant biochemical changes as in beef stored in the cooled state at a temperature of 0 ±0.5 °C for 16 days. Therefore, we have experimentally substantiated the quantitative indicators of the content of psychrotrophic microorganisms on the surface of beef intended for storage in a cooled or frozen state. The proposed microbiological criteria will improve the safety of beef.

Keywords: psychrotrophic microflora; microbiological criteria; beef chilled; beef frozen; volatile fatty acids of meat

INTRODUCTION
Meat and meat products are a significant part of a person's ration as they are a source of complete proteins. Due to its high nutritional value, meat is a favorable environment for the development of microorganisms **(Alonso-Hernando, Alonso-Calleja and Capita, 2013; Dave and Ghaly, 2011; Gunvig, Hansen and Borggaard, 2013; Lerma et al., 2015)**. Therefore, during its storage, short-term (cooling), long-term (freezing), or long-term (freezing), different temperature regimes are used to stop microbiological and physico-chemical processes **(Bruckner et al., 2012; Jeremiah, 1997; Pennacchia, Ercolini and Villani, 2011; Pothakos et al., 2014)**. According to the definition of the British Institute of Research in the field of food technology (UK Institute of Food Science and Technology, IFST) the storage date of the food is the time during which the product remains safe, ie, meets all the proper organoleptic, chemical, physical, microbiological properties, as well as food and nutritional requirements.

The beef and half carcasses of the beef are kept cool at 0 to -1 °C at 85% relative humidity for 16 days. Freezing involves storing of beef meat at -2 to -3 °C at 90% relative air humidity for 20 days, and freezing at -12, -18, -20, -25 °C at 95% relative humidity for 8 months, 12 months, 14 months. and 18 months, respectively standard DSTU 6030:2008 **(DSTU, 2009)**.

In the **Commission Regulation (EC) No. 2073/2005** and DSTU 6030:2008 **(DSTU, 2009)** of beef and veal in carcasses, half-carcasses and quarters specify the parameters and terms of refrigerated storage of beef and veal, microbiological standards for the safety of meat in excess of which indicate the need to improve the hygiene of slaughtering and review of measures to control the technological process. However, even within the standard temperatures of refrigerated storage of meat, there is different intensity of reproduction for certain groups of microflora **(Bruckner et al., 2012; Casaburi et al., 2014; Cerveny, Meyer and Hall, 2009; Zhang et al., 2019)**.

Therefore, even if the microbiological parameters meet the standard requirements before cooling, freezing, or chilling the meat, they may well exceed these standards at the end of the storage period.

Normally, after animals slaughtering, micro-organisms should be detected only on the surface of the carcasses, this is due to exogenous contamination and meets the sanitary and technological requirements **(Bruckner et al., 2012; Kameník, 2013; Salata et al., 2017; Serraino et al., 2012)**. According to **Commission Regulation (EC) No. 2073/2005**, the number of colonies of aerobic mesophilic micro-organisms on cattle carcasses before cooling should be from log 3.5 CFU.cm^{-2} to log 5.0 CFU.cm^{-2} area, and the content of bacteria of *Enterobacteriaceae* ranges from log 1.5 CFU.cm^{-2} to log 2.5 CFU.cm^{-2}.

Scientific publications and regulatory documents pay more attention to the contamination of beef carcasses, mainly mesophilic aerobic facultative anaerobic microorganisms and bacteria of the genus *Enterobacteriaceae*, which are indicators of compliance with sanitation during the slaughter of animals **(Cantalejo, Zouaghi and Pérez-Arnedo, 2016; Commission Regulation (EC) No. 2073/2005; Leroy et al., 2009; Nyamakwere et al., 2016)**.

It is believed that microbiological changes in meat occur due to the reproduction of psychrotrophic microflora when stored beef in a cooled and frozen state. **(Ercolini et al., 2009; Nieminen et al., 2011; Pothakos, Samapundo and Devlieghere, 2012; Pothakos et al., 2014; Stellato et al., 2017)**.

However, in recent years there has been a tendency to increase consumption and use as raw material for the frosted meat food industry compared to frozen **(Kukhtyn et al., 2020; Wei et al., 2019; Zhang et al., 2019)**. In this regard, technologies are being advanced that aim to extend the term of storage of frosted meat without altering organoleptic, physico-chemical and microbiological parameters **(Hilgarth, Behr and Vogel, 2018; Adam, Flint and Brightwell, 2010; Kukhtyn et al., 2019; Moschonas et al., 2011; Robertson et al., 2013)**. However, the term of storage of any product cannot be implemented in the production technology without a comprehensive scientific justification for all parameters that influence safety.

Therefore, a properly selected storage regime should ensure the maximum term of storage of the food product without disturbing its organoleptic, physico-chemical, and microbiological characteristics. With this in mind, it is relevant to study the content of psychrotrophic microflora during refrigeration storage of beef.

The purpose of this work was to evaluate the storage methods for refrigerated and frozen beef by microbiological and chemical parameters and to suggest criteria for evaluating beef by the content of psychrotrophic microorganisms.

Scientific hypothesis

It is possible to use the psychrotrophic group of microflora to evaluate the hygiene of the technological process of cattle slaughtering and beef processing and the suitability of meat for storage in a cooled and frozen state.

MATERIAL AND METHODOLOGY

A sampling of beef and carcass washes was carried out at meat processing enterprises of Lviv and Ternopil region, preparation for the investigation was performed according to ISO 6887-1:2017 and ISO 6887-2:2017 **(ISO, 2017a; ISO, 2017b)**. One part of the beef (half-carcass) was stored in a refrigerated state at 0 ±0.5 °C for 16 days and the second in the frozen state at 2 – 3 °C for 20 days. At the beginning of the experiment (cooled beef) and in 8, 16 days of storage in the cooled state and 10 and 20 days of storage in the frozen state, samples were taken and microbiological and biochemical parameters were determined.

Microbiological investigations were carried out in the laboratory of the Stepan Gzhytskyj Lviv National University of Veterinary Medicine and Biotechnologies. To determine the number of psychrotrophic microorganisms was sown 1 cm^3 of flushing or its ten-fold dilutions in Petri dishes, poured 15 cm^3 of molten and cooled to 45 ±5 °C MPA, incubation of crops was carried out at a temperature of +7.0 ±0.5 ºC for 10 days, and to determine aerobic mesophilic microorganisms, the crops were incubated at 30 ±1 °C for 72 ±1 h. The identification of pure cultures was performed according to the morphological, tinctorial, cultural, and biochemical properties, which are described in the Bergey bacteria determinant **(Vos et al., 2011)**. Tests were also used for the biochemical identification of non-fermenting microorganisms "Neferm test-24" (Rliva-lachema, Czech Republic).

The amount of volatile fatty acids was determined by a method based on the isolation of volatile fatty acids, which are accumulated in the meat during storage and determination by the titration amount of the distillate obtained with a solution of caustic potassium (or caustic soda). Herewith meat was considered fresh in terms of volatile fatty acids 4.0 mg KOH; – doubtful freshness – from 4.1 to 9.0 mg KOH; stale more than 9.1 mg KOH. The content of lipid peroxidation products in beef meat was determined by conventional methods, so the concentration of TBK-active products in tissue homogenates was determined by the method of Korobeinikova, 1989. To precipitate proteins to 1 mL of tissue homogenate was added 4.5 mL of 20% phosphoric acid and the samples were centrifuged. The supernatant was drained and 1.0 mL of 0.8 th thiobarbituric acid (TBK) solution was added to the precipitate and was kept for 1 h in a water bath at 100 °C. The tubes were then cooled and centrifuged. In the obtained centrifuge, the absorbance was measured at 535 and 580 nm against a control sample that contained bidistilled water instead of the homogenate. Double absorption measurement eliminates the absorption of colored complexes of thiobarbituric acid by substances of non-lipid nature. The content of TBK-active products was calculated by regression equation:

$$C = 0.21 + 26.5\Delta D$$

where C is the concentration of TBK-active products; ΔD – indicator D535 – D580 in the centrifuge. Diene conjugates (DCs) in the meat were determined spectrophotometrically.

Statistical analysis

Statistical processing of the results was carried out using methods of variation statistics using the program Statistica

9.0 (StatSoft Inc., USA). Non-parametric methods of research were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x) and the standard error of the mean (SE) were determined. The difference between the comparable values was considered to be significant for $p < 0.05$.

RESULTS AND DISCUSSION

The results of research on the dynamics of the microflora of beef meat cooled during storage are shown in Figure 1.

As can be seen from Figure 1, that in compliance with all veterinary and sanitary requirements for the procurement of beef meat in meat processing plants, the microbiological indices of the meat meet the established standards of the Regulation EU No 2073/2005 (permissible content of mesophilic micro-organisms up to 5 log CFU.cm^{-2} of carcass surface). In 8 days of storage at 0 °C, the total number of mesophilic microorganisms on the surface of half-carcasses was increased into 16.6 times ($p < 0.05$), and in 16 days – into 3 350 times ($p < 0.05$) and exceeded the allowed level following the regulations by 1.3 times and 258 times, respectively. It can also be noted that the content of psychrotrophic microorganisms was increased into 350 times ($p < 0.05$) in 8 days of meat storage and 52 thousand times ($p < 0.05$) in 16 days. If you compare the content of psychrotrophic microorganisms with the number of mesophilic during the process of meat storage, you can find the following. Psychrotrophic microorganisms on the surface of the chilled meat are 12.4 times smaller than mesophilic, but due to the faster rates of reproduction at this temperature, their number in the eighth day of storage was already 1.7 times ($p < 0.05$) higher. Psychotrophic microorganisms of chilled meat during the storage process have been the main dominant microflora, and this indicates its major role in the occurrence of microbiological defects in meat.

In Figure 2 results are given due to the microbiological investigations on the dynamics of microflora during the storage of beef in the frozen state at temperatures of -2 to -3 °C for 20 days.

As can be seen from Figure 2, that the content of mesophilic microorganisms beef meets the established requirements for 20 days of storage at temperatures of -2 to -3 °C. A decrease of 1.3 times ($p < 0.05$) in the number of mesophilic bacteria were detected after 10 days of storage, and after 20 days of their content remained practically unchanged. This does not indicate that the storage of meat in the frozen state inhibits or completely stops the development of mesophilic microorganisms for 20 days. At the same time, psychrotrophs, which can withstand low ambient temperatures, under these conditions increased their number on the surface of the beef in 10 days of storage into 4.5 times ($p < 0.05$). During the next 10 days of storage, that is, for 20 days, their number was increased into 7.9 times ($p < 0.05$) and amounted to 5.3 log CFU.cm^{-2} of surface area.

To fully characterize the microbiological changes in frozen meat, we determined the generic composition of psychrotrophic microflora, which is dominant in the storage process of beef at low temperatures.

In Figure 3 the composition of the psychrotrophic microflora of chilled beef is shown.

The identification of psychrotrophic microflora revealed that most of the cooled meat were bacteria of the genus *Acinetobacter* spp. – 55.1 ±2.2% and the smallest 14.6 ±0.7% of *Pseudomonas* spp. At the same time, an increase in the number of detected bacteria in the composition of psychrotrophic microflora of chilled beef was detected after 16 days of storage. Among the already identified three genera from the beef are bacteria of the genus *Flavobacterium* spp. and *Aeromonas* spp., which were not identified in the cooled meat, their number was 1.7 ±0.2% and 1.4 ±0.1%, respectively. This indicates the development of these bacteria during the storage of the beef in a chilled state. It is also seen that bacteria of the genus *Acinetobacter* spp. represent almost half of all psychrotrophic microflora of the cooled and chilled meat after 16 days of storage – 55.1 ±2.2 and 42.4 ±1.7% respectively. Bacteria of the genus *Alcaligenes* spp. occupy a stable niche of microflora, both cold and frozen meat – from 30.3 to 26.2%. However, bacterial growth of the genus *Pseudomonas* spp. Into 1.9 times ($p < 0.05$) on the surface of chilled beef, compared to cooled was observed.

Identification of the composition of psychrotrophic microflora of frozen beef after twenty days of storage (Figure 4) revealed an increase in bacteria of the genus *Pseudomonas* spp. into 1.3 times. At the same time, bacteria of the genus *Alcaligenes* spp. were consistently high in both cold and frozen meat – 30.3 – 31.7% respectively. Half of all psychrotrophic microflora accounted for bacteria of the genus *Acinetobacter* spp. 55.1 – 48.6%.

The research of the chemical indices of refrigerated and frozen beef during storage is shown in Figure 5.

As can be seen from the data of Figure 5, that the beef, which was stored in chilled state for 8 days in the content of volatile fatty acids, was of dubious freshness, indicating the beginning of the fat hydrolysis process. After 16 days of chilled beef storing at 0 ±0.5 °C, a certain indicator indicates the deterioration of the meat. In particular, the number of volatile fatty acids was increased into 5.1 times ($p < 0.05$), indicating that the course of intensive biochemical processes of enzymatic hydrolysis of fat and beef with such indicators is characterized as not fresh.

Thus, researches indicate that storing beef in chilled state at 0 ±0.5 °C for more than eight days is impractical, as its chemical characteristics are getting worse and there are signs of spoilage.

Research of beef stored in a frozen state at the temperature -2 to -3 °C revealed that, after 10 days, the number of volatile fatty acids remained at the level characteristic of fresh meat. In 20 days of meat storage, an increase in the amount of volatile fatty acids was found to be 2.3 times ($p < 0.05$). According to these indicators, meat is characterized as doubtful freshness.

Therefore, storage of beef in the frozen state at a temperature of 2 to -3 °C for 20 days does not cause such significant biochemical changes as in beef stored in the cooled state at a temperature of 0 ±0.5 °C for 16 days.

The next part of our research was to determine the content of lipid peroxidation (POL) products in chilled and frozen beef. It is well known **(Vega et al., 2009)** that the content of POL in meat is increasing with prolonged storage, which negatively affects its quality – smell, taste, structure. The results of researches on the content of TBK-active products (TBKAP) and diene conjugates (DCs) in chilled beef during storage are shown in Figure 6.

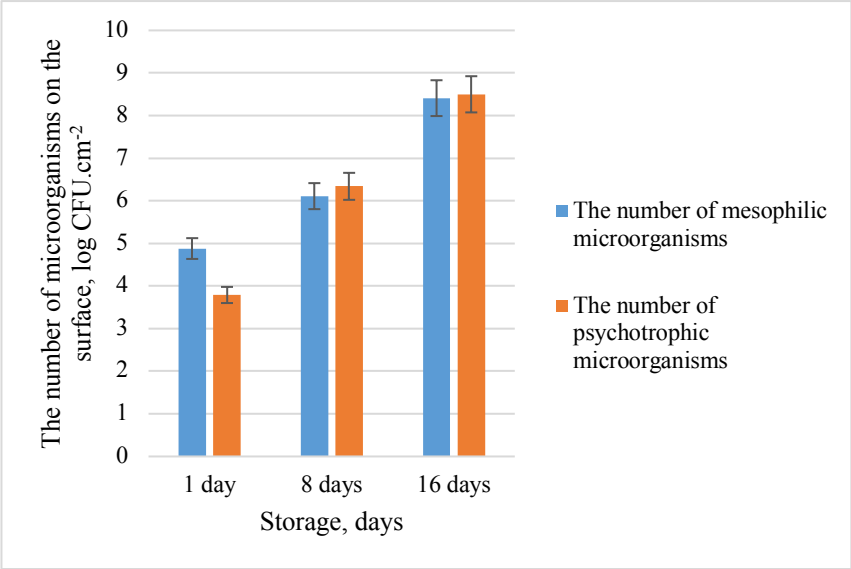

Figure 1 Changes of mesophilic and psychrotrophic microflora during storage of beef in a cooled state at a temperature of 0 ±0.5 °C.

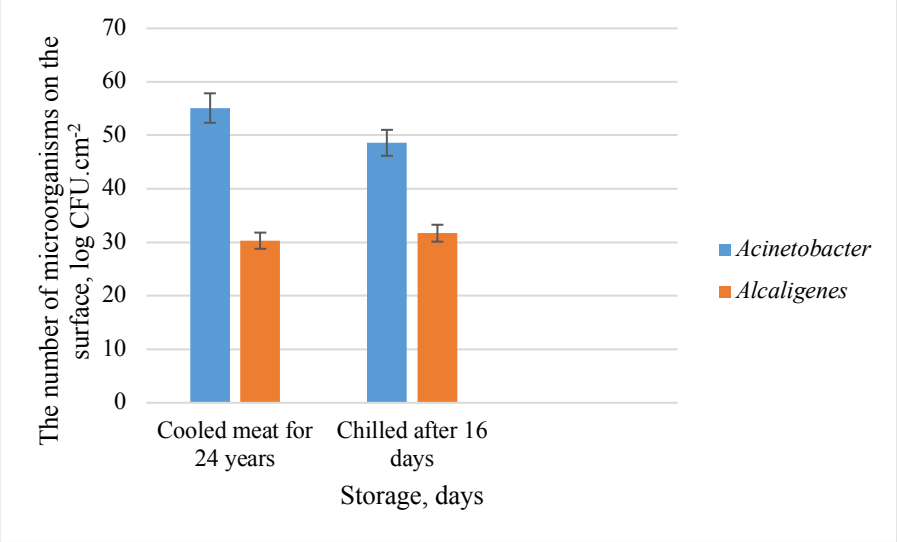

Figure 2 Changes of mesophilic and psychrotrophic microflora during storage of beef in the frozen state at temperatures -2 to -3 °C.

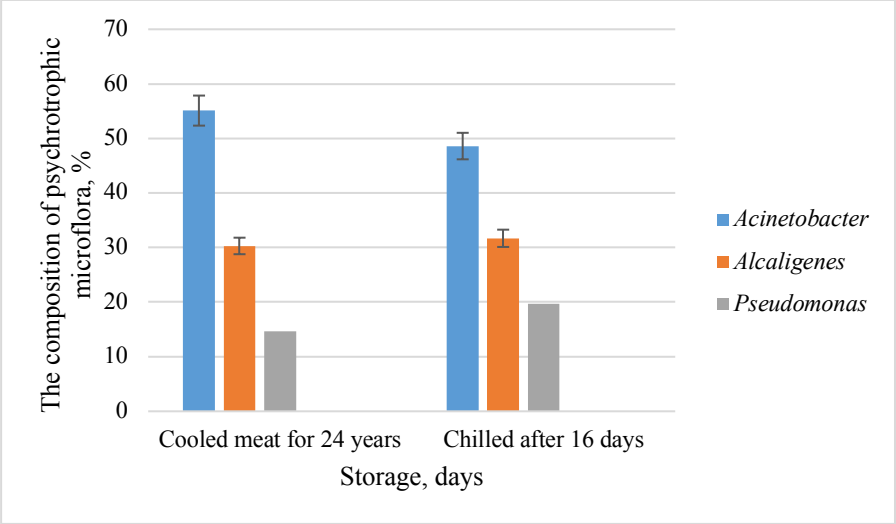

Figure 3 Generic composition of psychrotrophic microflora of beef during storage in a cooled state at a temperature of 0 ±0.5 °C.

As can be seen from Figure 6, that when the meat was kept refrigerated at 0 ±0.5 °C, no significant increase in the amount of TBKAP and DC on the eighth day was observed. On the 16th day of storage, there is a probable increase (p <0.05) in the number of TBKAP and DC compared to the first day.

When storing beef in the frozen state at a temperature of -2 to -3 °C, a probable increase into 1.3 times (p <0.05), compared to the first one, was noted only by the amount of DC for 20 days (Figure 7).

Thus, the obtained data are shown in Figure 6 and Figure 7 indicate that as the temperature of the refrigeration treatment of beef decreases, its resistance to oxidation increases, in particular the growth of TBKAP and DC.

The results indicate that the initial amount of microflora, especially the content of psychrotrophs, is crucial for the choice of beef storage temperature. Therefore, we are offered to evaluate the suitability of beef for storage in a cooled and frozen state according to the following hygiene criteria of the technological process (Table 1).

If a microbiological investigation of five beef samples before cooling from one batch reveals at least one sample of psychrotrophic microorganisms over 4 log CFU.cm^{-2} of area (≥M), then such beef is used immediately, and measures are taken to improve the hygiene of the technological process.

If three beef samples are detected, the number of psychrotrophic microorganisms from 3 log CFU.cm^{-2} area to 4 log CFU.cm^{-2}, (between m and M), then keep such batch in the cooled state at a temperature of 0 ±0.5 °C for not more than 8 days, or in the frozen state at a temperature of 2 to -3 °C for up to 20 days.

If a microbiological examination of five beef samples reveals a number of psychrotrophic microorganisms less than 3 log CFU.cm^{-2} of area (m), then such a batch is stored in chilled state at 0 ±0.5 °C for up to 16 days, or in the frosted state for 2 – 3 °C up to 20 days.

Thus, the proposed criteria for evaluating beef before storage allowed to scientifically justify the optimal cooling or freezing temperature to obtain, at the end of the storage period, meat with satisfactory organoleptic, physico-chemical and microbiological parameters.

The urgency of the issue of fresh meat and increasing the storage term is the primary purpose for meat industry professionals. Meat has a limited storage term, creating difficulties for both producers and consumers for whom a defective product is potentially dangerous **(Jeremiah, 1997; Nyamakwere et al., 2016)**. The biggest factor which causes spoilage of meat during its storage is microbiological **(Bruckner et al., 2012; Casaburi et al., 2014; Cerveny, Meyer and Hall, 2009; Pothakos, Samapundo and Devlieghere, 2012; Zhang et al., 2019)**. Therefore, first of all, it is necessary to minimize contamination by microorganisms from the moment of slaughter to processing and to inhibit the development of existing microflora through the use of refrigeration **(Bruckner et al., 2012; Dave and Ghaly, 2011; Kameník, 2013; Serraino et al., 2012)**. However, even during refrigerated storage (cooling, freezing) of beef meat, it is spoiled by the reproduction and activity of psychrotrophic microflora. **(Bruckner et al. 2012; Jeremiah, 1997; Pennacchia, Ercolini and Villani, 2011; Pothakos et al., 2014)**. Existing regulatory documents control the hygiene of the technological process of beef only by the content of mesophilic aerobic microorganisms and bacteria of the *Enterobacteriaceae* genus **(Commission Regulation (EC) No. 2073/2005)**.

Our investigations have found that the number of mesophilic microorganisms on fresh (24 h) beef carcasses was 12.4 times higher than the number of psychrotrophic microorganisms. During storage of the beef in chilled state at temperature 0 °C for 8 days, the number of aerobic mesophilic microorganisms was increased on the surfaces of the half-carcass by 16.6 times, and in 16 days – into 3 350 times and exceeded the admissible level (up to 5 log CFU.cm^{-2} of the carcass surface) into 1.3 times and 258 times respectively. At the same time, the psychrotrophic microflora during this period of storage increased 350 times in 8 days and in 52 thousand times in 16 days. That is, on the 8th day, the amount of psychrotrophic microflora already in 1.7 times outweighed the content of mesophilic microflora. In research **(Bruckner et al, 2012; Ercolini et al., 2009; Hassan et al., 2015; Pothakos, Samapundo and Devlieghere, 2012; Pothakos et al., 2014)** was also found a higher content of psychrotrophic microorganisms in chilled foods compared to the number of mesophilic aerobic bacteria. Researchers consider the use of mesophilic aerobic microorganisms as a parameter for estimating the storage term of chilled foods rather dubious **(Maas van Berkel, van den Boogaard and Heijnen, 2004; Hilgarth, Behr and Vogel, 2018; Salata et al., 2017; Zhou, Xu and Liu, 2010)**.

Overall, our results indicate that storage of beef meat with an initial mesophilic bacterial content of about 4.88 log CFU.cm^{-2} of surface and psychrotrophic bacteria 3.79 log CFU.cm^{-2} at temperature 0 °C is only possible for 8 days, further, the microbiological indices exceed the acceptable standards and half-corpuscles are unusable. Therefore, we believe that when storing chilled meat at temperature 0 °C, it is necessary to achieve a reduction in the initial inoculation of the carcasses by microorganisms by improving the sanitation conditions of meat provision at meat processing plants.

Investigation of the dynamics of microflora reproduction during the storage of beef in the frozen state at temperature -2 to -3 °C for 20 days established a decrease in 1.3 times the number of mesophilic bacteria in 10 days of storage. At the same time, the number of psychrotrophic microorganisms during this storage time was increased in 4.5 times, and 20 days in 7.9 times and amounted to 5.3 log CFU.cm^{-2} of surface area. This indicates that the storage of meat in the frozen state inhibits or completely stops the development of mesophilic microorganisms for 20 days. Therefore, we support the opinion of scientists **(Alonso-Hernando, Capita and Alonso-Calleja, 2013; Bruckner et al., 2012; Cerveny, Meyer and Hall, 2009; Jeremiah, 1997; Kameník, 2013; Pennacchia, Ercolini and Villani, 2011; Pothakos et al., 2014)**, which indicate that the temperature of refrigeration processing of meat has a significant influence on its storage term.

Thus, even though the meat complies with the standards by the content of mesophilic bacteria, the presence and development of psychrotrophic microorganisms in frozen meat is an integral part of the safety and quality control of beef.

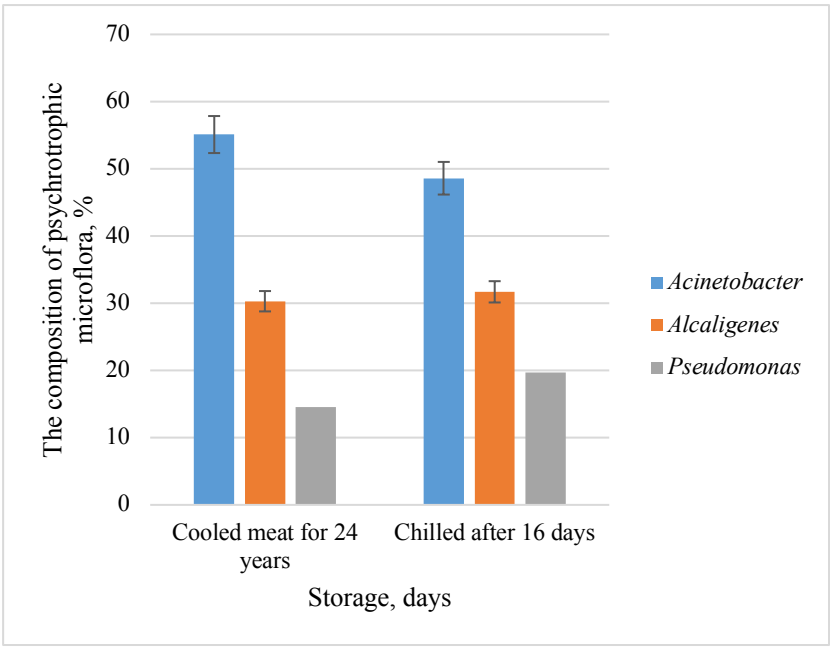

Figure 4 Generic composition of psychrotrophic microflora of beef during storage in the frozen state at temperatures -2 to -3 °C.

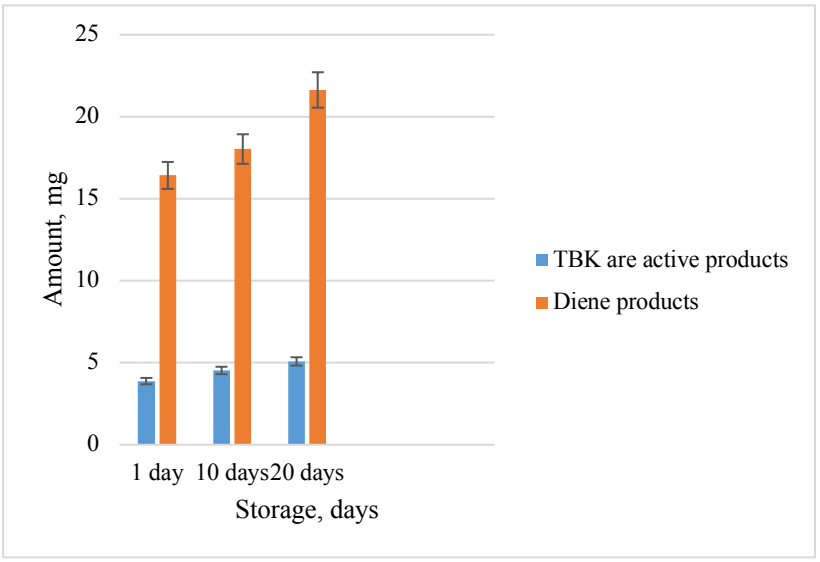

Figure 5 Changes in the content of volatile fatty acids during storage of beef in a cooled and frozen state at temperature. Note: KOH; – doubtful freshness – from 4.1 to 9.0 mg KOH; stale more than 9.1 mg KOH.

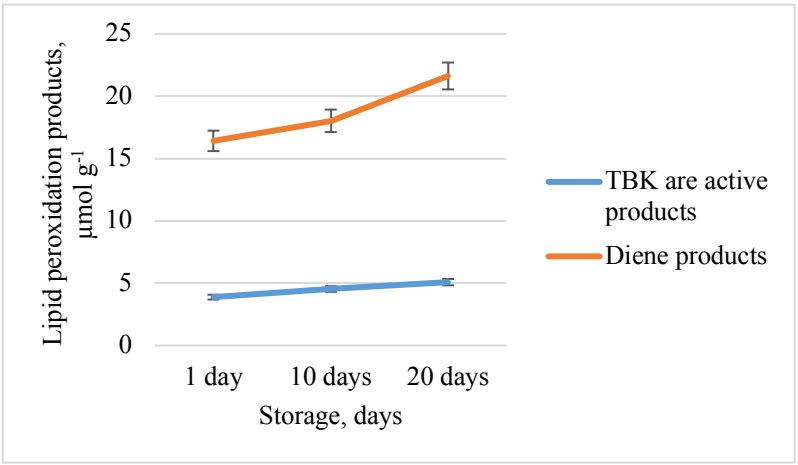

Figure 6 Changes in lipid peroxidation products during storage of the beef in chilled state at 0 ±0.5 °C.

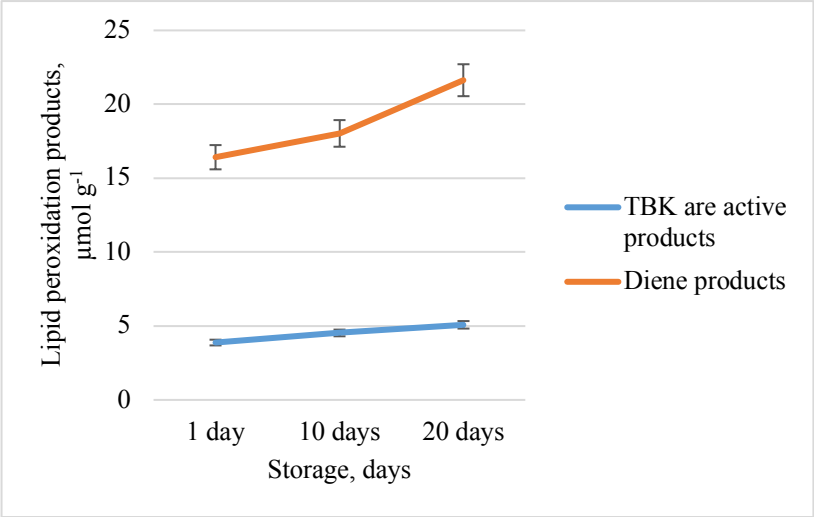

Figure 7 Changes of lipid peroxidation products during the storage of beef in the frozen state at temperatures -2 to -3 °C.

Table 1 Microbiological evaluation of chilled and frozen beef according to by the criteria of technological hygiene processes for the determination of psychrotrophic microflora.

Food category	Microorganisms	Sampling plan		Permissible limits		The stage where the metric is applied	Actions in case of poor results
		n[1]	c[2]	m[3]	M[4]		
Beef meat	Psychrotrophic	5	3	3 log CFU.cm^{-2}	4 log CFU.cm^{-2}	Before staging for refrigerated storage	improvement of slaughter hygiene and reviewing process control measures

Note: [1]n – number of samples taken from one carcass; [2]c – number of samples, parametric values, which are between *m* i *M*; [3]m – normative value of the content of microorganisms per 1 cm^2 of the carcass surface; [4]M – maximum content of microorganisms per 1 cm^2 of the surface.

An important factor that depends on the appearance of organoleptic defects in meat during its refrigerated storage is the microbial composition of the existing microflora because the production of aromatic substances and the decomposition of proteins, fats, and carbohydrates in different genera and species of microorganisms are different **(Cerveny, Meyer and Hall, 2009; Ercolini et al., 2009; Kameník, 2013; Adam, Flint and Brightwell, 2010; Pothakos et al., 2014; Stellato et al., 2017; Zhang et al., 2019)**. When identifying the psychrotrophic microflora of beef, it was found that bacteria of the genus *Acinetobacter* spp. make up almost half of all psychrotrophic microflora cooled and chilled after 16 days of storage 55.1 ±2.2 and 42.4 ±1.7% respectively. Bacteria of the genus *Alcaligenes* spp. occupy a stable niche of microflora, both cold and frozen meat – from 30.3 to 26.2%. However, bacterial growth of the genus *Pseudomonas* spp. was observed n 1.9 times on the surface of chilled beef, compared to cooling.

The identification of the composition of the psychrotrophic microflora of frozen beef after twenty days of storage revealed an increase in bacteria of the genus *Pseudomonas* spp. in 1.3 times. At the same time, the number of bacteria of the genus *Acinetobacter* spp. and *Alcaligenes* spp. in frozen meat was almost the same as in the cold. In research **(Doulgeraki et al., 2012; Ercolini et al., 2009; Stellato et al., 2017)** it is reported that the most responsible for the emergence of food defects during refrigeration storage are bacteria of the genus *Pseudomonas* spp. However, in large quantities stand out such psychrotrophic genus as *Acinetobacter* spp., *Brochothrix* spp., *Flavobacterium* spp., *Psychrobacter* spp., *Moraxella* spp., to a lesser extent *Staphylococcus* spp., and *Micrococcus* spp., lactic acid bacteria and genus Enterobacteriaceae **(Dave and Ghaly, 2011; Kameník, 2013; Pennacchia, Ercolini, and Villani, 2011)**.

Therefore, investigations indicate that bacteria of genus *Pseudomonas* spp. during the storage of beef in the cooled and frozen state show the highest intensity of development.

An assessment of the volatile fatty acid content of chilled beef found that after 8 days of storage, the meat was of dubious freshness, and after 16 days it was not fresh. In particular, the amount of volatile fatty acids was increased in 5.1 times by 16 days, indicating the course of intensive biochemical processes on enzymatic hydrolysis of fat and spoilage of meat. An investigation of frozen beef was found that after 10 days the amount of volatile fatty acids did not increase, and on the 20th day their storage their number was 2.3 times higher compared to the content in the cooled meat. With so many volatile fatty acids, the meat is considered to be of doubtful freshness.

Literary data **(Ercolini et al., 2009; Jay, Loessner and Golden, 2005; Mayr et al., 2003)** also indicate that during the storage process of meat and the development of the

microflora, there is a release of various aromatic compounds that cause organoleptic defects of meat. Therefore, we support the opinion of scientists that the amount of volatile fatty acids increases due to the microbial metabolism of psychrotrophic microflora **(Morales, Fernández-García and Nuñez, 2005; Padda et al., 2001; Popelka, Jevinová and Marcinčák, 2016)**.

CONCLUSION

Our investigations found that while storing meat in a refrigerated state, a probable increase in the amount of TBKAP and DC was detected by only 16th day compared to the first one. When storing beef in the frozen state, a probable increase in 1.3 times, compared to the first day, was noted only by the amount of DC on the 20th day. This indicates that active oxidation of polyunsaturated fatty acids in cell membrane phospholipids occurs in chilled beef meat after the eighth day of storage.

Thus, the investigations indicate that storing of beef in the cooled state at a temperature of 0 ±0.5 °C for more than eight days is impractical, as its biochemical indices are worsening and signs of spoilage are appearing. At the same time, storing of beef in the frozen state at a temperature of -2 to -3 °C for 20 days does not cause such significant biochemical changes as in beef stored in the cooled state at a temperature of 0 ±0.5 °C for 16 days. Also, to prevent the occurrence of organoleptic and biochemical beef defects during refrigeration, it is necessary to stop the development of psychrotrophic proteolytic and lipolytic microflora, which is possible by lowering the temperature.

Therefore, we have experimentally substantiated the quantitative indicators of the content of psychrotrophic microorganisms on the surface of beef intended for storage in a cooled or frozen state. The proposed microbiological criteria based on European approaches will improve the safety of beef.

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Influence of maintenance technology in arid conditions on efficiency of marbled beef production

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ABSTRACT
In recent years, Russia has been developing domestic single-purpose meat cattle breeding. Some livestock farms have focused on the production of high-quality grades of raw meat, i.e. 'marbled' beef. Meat of that kind is in great demand in the premium meat market. At the same time, production of beef without using steroids and hormonal drugs increases the competitiveness of this product on the world market in countries of the West and the Middle East. Within the framework of our study, an experiment was conducted on Kalmyk cattle in the arid conditions of OAO Kirovskij in the Republic of Kalmykia. Standardized methods of analysis were used. For the experiment, 10-month-old steers were selected and divided into two groups (Control and Test), 30 heads each. They were kept and fed according to different technologies until the age of 19 months. The Test group steers were kept tied up and fed with a diet developed by the Volga Region Research Institute of Manufacture and Processing of Meat-and-Milk Production; the Control group steers were kept according to conventional beef cattle production technology. The wide expansion range and popularity of this cattle breed are caused by the high productivity of steers whether kept tied up or allowed to graze free. The compared qualities of beef obtained from Kalmyk steers proved that keeping them tied up allows increased production efficiency of raw meat which is an important factor for meat production, being intensified in an unstable situation in agriculture.

Keywords: beef; feeding technology; grass-fed; 'marbled'; meat cattle breeding

INTRODUCTION
Meat is of great importance for the human diet, as it is a source of complete animal protein **(Avilés et al., 2015; Johnson et al., 2019)**. Research shows that most high-quality and balanced meat is obtained from cattle of meat breeds grown according to special fattening technology for the production of 'marbled' meat grades **(Culbertson et al., 2015; Kenny et al., 2018; Shumilina, Skvortsova and Grebenshchikov, 2012)**. The meat of various animal species can be marbled, i.e. cattle, small ruminants and pigs **(Belyaev et al., 2004; Burrow, 2001; Kayumov, 2008)**. The prospects of beef production and modern technologies for keeping and feeding cattle of different breeds in different regions of Russia and elsewhere in the world have been developed, investigated and scientifically substantiated by some scientists **(Adeyemi et al., 2019; Kelly et al., 2019; Lindholm-Perry et al., 2017; McIvor and Monypenny, 1995)**.

According to the literature, the Kalmyk breed has a genetic potential for the formation of marbled beef and is best adapted for the arid territories of southern Russia. Steers of this breed are widespread and popular in Russia due to their adaptive abilities **(Randelin et al., 2018)**. Currently, the Kalmyk breed of cattle is grown in 34 regions of Russia, i.e. in the regions of the North Caucasus, Southeast, Volga, Urals and the Far East.

The above-mentioned advantages of the Kalmyk breed caused its expansion into areas with severe agro-climatic conditions: Siberia, the Caucasus and Transbaikalia. Kalmyk cattle are kept and bred most often on pastures in natural habitat.

However, in recent years, there has been an insufficient number of pasture lands on farms, especially in regions with a severe harsh continental climate and features of desert invasion. For this reason, beef cattle are kept and raised tied up. In the absence of large pasture resources, this technology allows the production of raw meat in arid territories. At the present stage of cattle breeding, scientific investigations of the Kalmyk breed and the quality characteristics of the beef obtained do not pay attention to comparing the technologies under which livestock are kept: tied up or in free stalls. This led us to a scientific experiment aimed at comparing the productivity and quality characteristics of beef obtained by the technologies under which steers are kept and fattened either tied up or in free stalls in the arid conditions of the Republic of Kalmykia.

Scientific hypothesis

The maintenance technology and feeding animals with a specially formulated feed contributes to the rapid maturation and marbling of beef.

MATERIAL AND METHODOLOGY

Ethical approval

The authors confirm that the studies were conducted in accordance with internationally recognized ethical standards of the Helsinki Declaration on clinical researches.

Experimental design

The study was conducted on the territory of a farm in the South of Russia. We studied two groups of bull calves that were raised using different feeding and keeping technologies. An experiment was conducted to assess the process of forming marbled meat in the arid conditions of the Republic of Kalmykia. For this purpose, 60 animals of the Kalmyk breed, formed into two groups (experimental and control), were selected from the Kirovsky livestock farm. The animals of the control group were kept and fed according to the traditional technology without binding and on pastures, while the experimental group received green feed mown on specially designated areas of pasture on the farm and were kept on a leash in stalls. The rations for the experimental animals were developed using the Feed Optima Expert program by the standards and age.

Research materials were processed using graphical, trend and statistical analysis methods, as well as using the Microsoft Office software package. The paper uses generally accepted standardized methods of analysis of the studied objects.

Dynamics of live weight, and meat yield

The dynamics of live weight were studied by weighing experimental animals, calculating the absolute and average daily growth, and slaughter indicators: carcass weight, pulp, slaughter yield, etc., according to approved methods. The morphological composition of carcasses was studied by cutting them into cuts according to state standard no. 31797-2012 'Meat. Dressing of beef into cuts. Specifications'. To determine the quality indicators, average samples of the pulp and the longest back muscle were then selected. The meat index has the following formula:

$$\frac{\text{half - girth of the back}}{\text{height at the withers}} * 100\%$$

Histological analysis

Microscopy of samples of the longest back muscle of experimental bulls was performed using an electron microscope (Zeiss AxioImager.Z2), synchronized with a personal computer, having previously prepared a sample-a tool for preparing sections of a fixed 'Microtome' with the MS-1 system ('MicroM').

Statistical analysis

The experimental data on different variables were statistically analysed using the Statistica 10 package (StatSoft Inc.). The significance of differences between the indices was determined using the criteria of nonparametric statistics for the linked populations (differences with p <0.05 were considered significant: ***p <0.001; **p <0.01; *p <0.05; ns = not significant at p >0.05). Student's t-test was applied for the statistical analysis.

RESULTS AND DISCUSSION

Domestic and foreign scientists have shown the relevance of a comprehensive study of raw meat production technology in natural pasture conditions **(Asp et al., 2012; Fedotova et al., 2019; Shumilina, Skvortsova and Grebenshchikov, 2012; Troy, Tiwari and Joo, 2016)**.

The results of the experiment showed that steers gained live weight differently during the observation period from the 10th to 19th months of age, depending on the keeping and feeding technology.

It was determined that marbled beef is obtained from animals that are kept in stalls for a long time. It is obvious that by adjusting the percentage of intermuscular fat by changing the nutritional content of diets, you can win more consumers by producing various products with the necessary characteristics; this is confirmed by modern research **(Basarab et al., 2003; Luo et al., 2019)**. Marbling also depends on the time of feeding and the type of feed. The longer the cattle are fed high-calorie food, the more likely it is that they will have higher quality indicators, but significantly less marbled meat (as a percentage of the carcass, that is, the ratio of lean meat to marbled meat). The period after setting the animals to fattening lasts approximately 3 – 4 months and if the diet is not balanced it can lead to gastrointestinal pathology, so it is not recommended that the period of rearing livestock is extended **(Gorlov, Mokhov and Vorontsova, 2018; Samikshya et al., 2019)**. Feeding with a large amount of cereals, such as corn or barley, will change the colour of the fat from yellowish to white. Low physical activity is also a factor that affects marbling. In cows and steers that have grown up in tight stalls, the meat becomes softer than in free-grazing animals. Thus, animals that are restricted in movement easily accumulate intramuscular fat, and their meat becomes soft **(Adeyemi et al., 2019; Troy, Tiwari and Joo, 2016)**. The worldwide accepted technology for growing and fattening cattle to produce marbled meat is feedlots – platforms for final fattening with high-calorie diets for at least 120 days before slaughter, before animals graze free **(El-Khadrawy, Ahmed and Hanafi, 2011; Gorlov, Mokhov and Vorontsova, 2018)**.

The live weight (kg) and growth dynamics in the experimental groups of steers are presented in Table 1.

Assessment of the weight gain rate of steers showed that live weight gain in the Test group was faster, which affected the weight dynamics in different months. According to the study results in Table 1, higher live weight dynamics were shown by the Test group steers kept tied up and fed with the special recipe fodder developed by the Volga Research Institute **(Gorlov, 2018)**.

Comparing the dynamics of average daily live weight gain (kg) in the groups enabled observation of the maximum growth energy in Test steers at the age of 16 to 18 months. Therefore, we recommend raising steers to 18 months old (Figure 1, Figure 2).

Table 1 Live weight (kg) dynamics of steers.

Age, months	Group	
	Free-stall (Control)	Tie-up (Test)
10	260.7 ±1.03	261.2 ±1.17*
11	284.9 ±1.13	285.4 ±1.83
12	311.7 ±1.54	313.6 ±1.66**
13	337.5 ±1.71	341.7 ±1.95
14	363.0 ±1.62	367.6 ±1.81
15	387.2 ±2.23	394.4 ±2.82
16	411.0 ±2.05	420.0 ±2.12*
17	433.4 ±2.18	444.8 ±1.77
18	455.4 ±1.97	467.9 ±2.33
19	476.1 ±2.25	489.1 ±2.71***

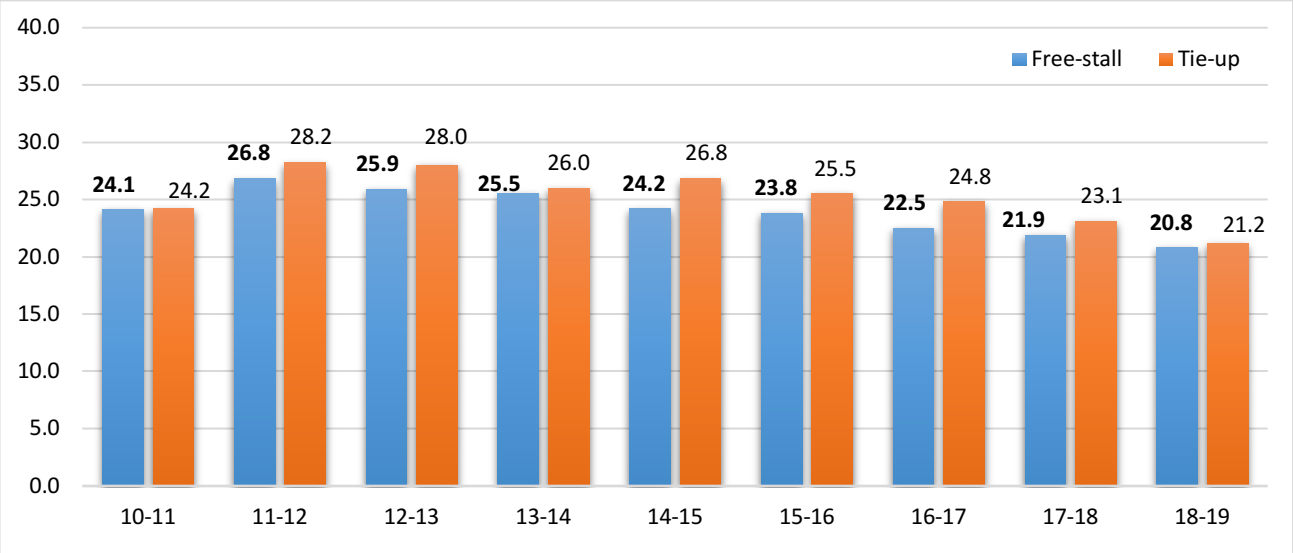

Figure 1 The overall live weight gain (kg) of experimental steers.

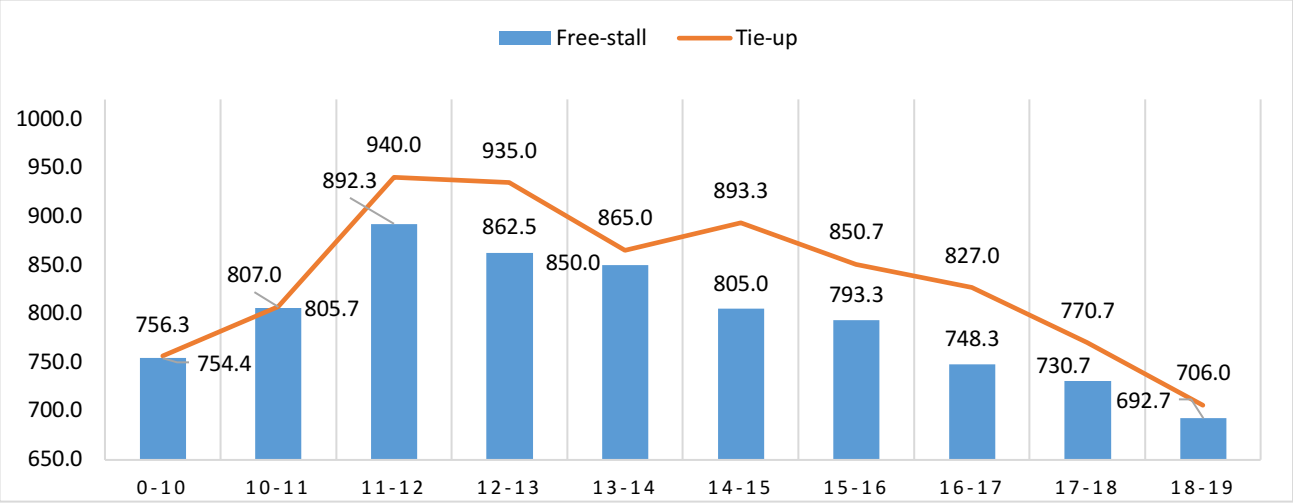

Figure 2 Average daily gain (g) of experimental steers.

Table 2 Control slaughter results of experimental steers.

Indicator	16 months		17 months		18 months		19 months	
	Free-stall (Control)	Tie-up (Test)	Free-stall (Control)	Tie-up (Test)	Free-stall (Control)	Tie-up (Test)	Free-stall (Control)	Tie-up (Test)
Pre-slaughter weight, kg	410.8 ±1.8	414.1 ±1.5*	431.6 ±1.8	436.5 ±1.8	449.7 ±1.8	453.9 ±1.95	471.0 ±1.6	474.1 ±2.2*
Carcass weight, kg	226.1 ±1.9	229.3 ±2.03	241.4 ±1.8	246.3 ±1.8*	253.9 ±1.8	258.1 ±1.8	269.1 ±1.8	272.3 ±1.8
Carcass yield, %	55.0	55.4	55.9	56.4	56.5	56.9	57.1	57.4
Slaughter weight, kg	237.6 ±1.5	240.8 ±1.7**	252.9 ±1.6	257.8 ±2.1	265.4 ±2.2	259.6 ±1.8	280.9 ±2.1*	284.1 ±1.8
Slaughter yield, %	57.8	58.2	58.6	59.1	59.0	59.4	59.7	59.9
Weight of chilled carcass, kg	210.4 ±1.4	213.6 ±1.8ns	225.7 ±1.8	230.6 ±2.0	238.2 ±2.1	242.3 ±1.6	253.4 ±1.6*	256.6 ±1.7
Flesh weight, kg	166.6 ±1.3	169.8 ±1.3	181.9 ±1.6	186.8 ±1.6	194.4 ±2.0	198.5 ±1.9	209.6 ±1.9	212.8 ±1.6*
Flesh yield, %	79.2	79.5	80.6	81.0	81.6	81.9	82.7	82.9

Table 3 Morphological composition of the carcasses of experimental steers.

	16 months		17 months		18 months		19 months	
	Free-stall	Tie-up	Free-stall	Tie-up	Free-stall	Tie-up	Free-stall	Tie-up
Subscapular	15.76	16.21	17.38	18.78	18.71	19.54	19.14	21.45
Neck	44.85	46.11	49.31	51.36	53.02	54.13	56.09	58.97
Hip cut on bone without shank	25.07	26.43	27.88	28.97	30.12	32.07	31.93	32.76
Shoulder blade	42.66	44.56	46.57	48.04	50.23	52.09	53.21	54.78
Sternocostal	76.06	78.14	83.77	84.59	90.19	91.26	95.85	98.43
Saddle	28.25	29.54	31.08	32.89	33.6	34.78	36.26	39.53

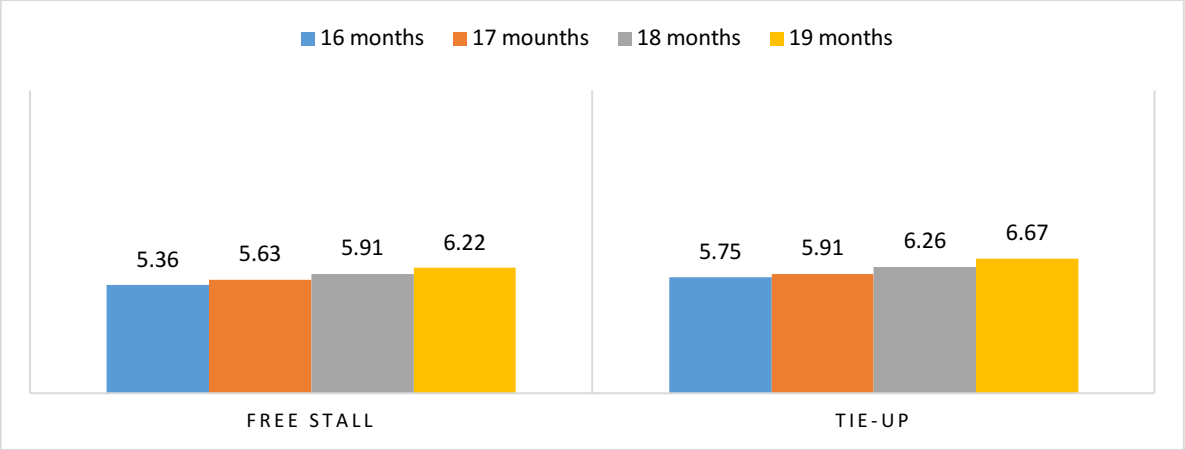

Figure 3 Dynamics of the meatiness index of experimental steers.

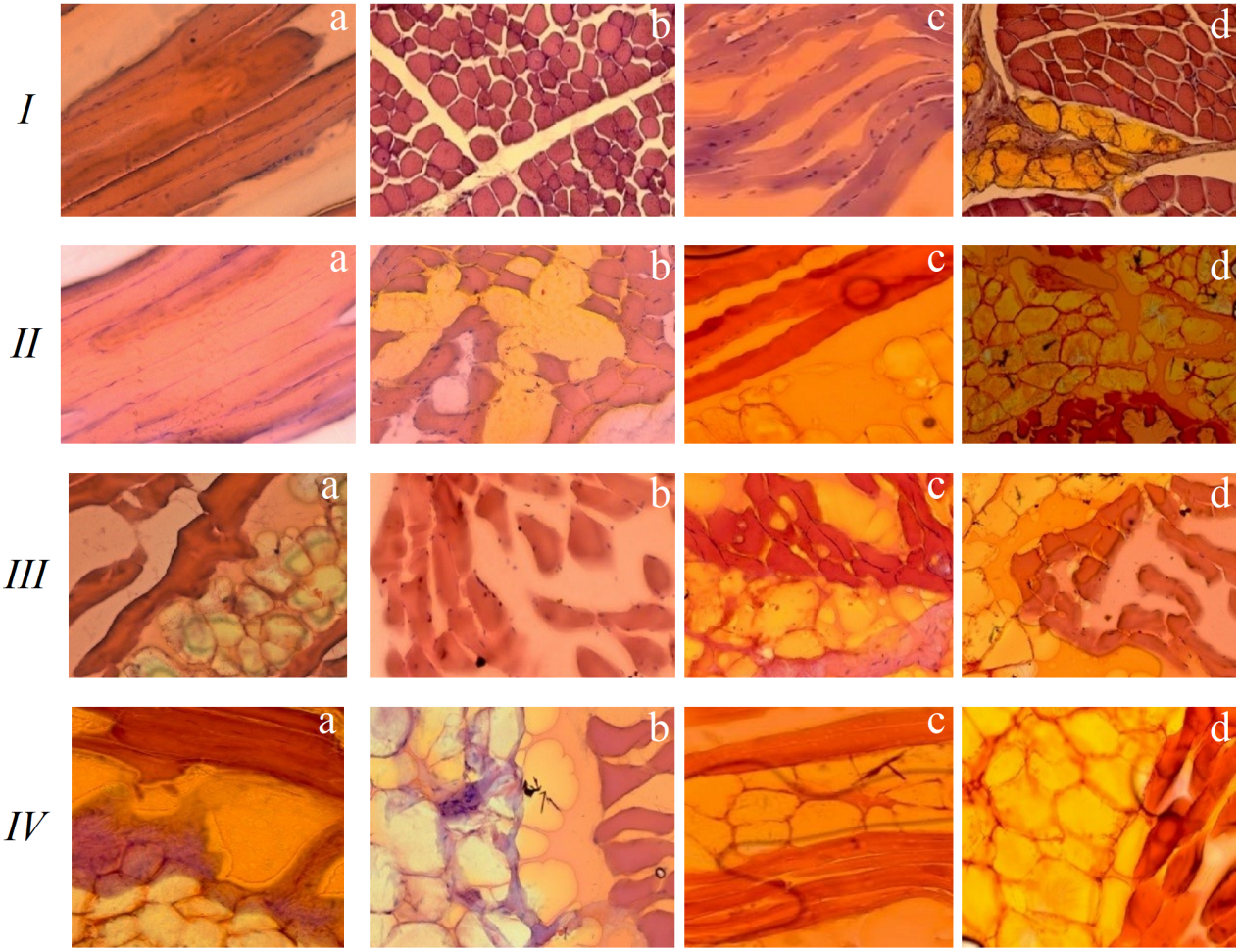

Figure 4 Microstructure of the *Longissimus* muscle of experimental steers at different ages: (I – 16, II – 17, III –18 and IV – 19 months; a, b – longitudinal section; c, d – cross section.

In the next stage of the experiment, steers at the age of 16, 17, 18 and 19 months were slaughtered in a slaughterhouse, to study the marble formation. The main indicators of the controlled slaughter of steers are presented in Table 2.

The control slaughters of experimental steers at different ages showed that the live weight dynamics in the Test group were higher than in the Control group by 8.3 – 10.5 kg, pre-slaughter weight by 8.5 – 11.3 kg, slaughter weight by 9.5 – 13.9 kg, chilled carcasses by 9.5 – 13.9 kg and flesh weight by 9.4 – 15.3 kg. The Kalmyk breed under consideration was characterized by high meatiness and hardiness with the meat index increasing for this keeping technology (Figure 3).

The morphological composition of the carcasses examined showed that in all steers, the most massive parts of the carcass are the hip cut, sternocostal and shoulder blade. In the Test group, these parts exceeded the weight of similar cuts of steers in the Control group (Table 3). Samples were taken from the rib-saddle part of Test steers to examine the microstructure of muscle tissue and assess the degree of marbling in the raw meat obtained. The results of the histological analysis are presented in Figure 4.

The muscle tissue samples were taken from animals at 16, 17, 18 and 19 months old in the Test group to establish the marbled structure of meat and its dynamic formation.

The unique ability of Kalmyk steers to accumulate reserve nutrients in the form of marbled layers gives high culinary qualities to their beef **(Prokhorov, Naumovich and Mulangi, 2016)**. Moreover, the value of this meat grade is due to its high content of vitamins and minerals, and a unique ratio between monounsaturated and saturated fats **(Makayev, 2005; Šulcerová et al., 2017; Yaremchuk and Rodin, 2011)**.

In assessing the microstructure of the muscle tissue, we found considerable fatty tissues in fibres, which characterized the degree of beef marbling. While some studies have shown that beef marbling is determined by the colour of muscle and fat tissue and marbling on the longest back muscle (*Longissimus dorsi*) of cattle **(Frank et al., 2016; Morozova, 2016; Yaremchuk and Rodin, 2011)**. Fat inclusions were found in the samples of all age groups, but the maximum marbling had been reached by 19 months; these results support some of the results of other studies **(Wang et al., 2019; Kayumov, 2008; Randelin et al., 2018)**.

Thus, it was proved that marbling is formed in the meat raw materials produced, depending on the feeding and keeping technology.

The tie-up keeping technology and feeding with a specially formulated feed mixture contributed to the early

maturation and marbling of beef. In addition, the proven experimental technology developed by the Volga Research Institute contributed to the rapid weight gain of steers and production of premium beef grades.

CONCLUSION

Single-purpose beef cattle breeding in the arid conditions of the South of Russia is the most promising direction for the development of agricultural production in these territories. The severe climatic conditions and waterless periods allow the breeding and keeping cattle of breeds best acclimatized to this area. The Kalmyk cattle breed grown in this area is the most promising in terms of the production of premium beef grades due to its genetic predisposition to marbling of the raw meat obtained. The wide expansion range and popularity of this cattle breed are caused by the high productivity of steers whether kept tied up or allowed to graze free. The compared qualities of beef obtained from Kalmyk steers proved that the tie-up keeping technology allowed increased production efficiency of raw meat.

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EFFECTS OF SELENIUM ON MACRO- AND MICRONUTRIENTS AND SELECTED QUALITATIVE PARAMETERS OF OAT (*AVENA SATIVA* L.)

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ABSTRACT

The article deals with the effect of foliar Se application on macro-and micro-elements and selected quantitative parameters (the content of ash, starch, and fat) in oat grains. The three-year experiments were carried out on Research and Breeding Station Vígľaš – Pstruša in the years 2014, 2015, 2016. The used oat variety was Valentin. The experiment was performed by a block method within a parcel size of 10 square meters (8 x 1.25 m) with the span of rows amounting to 0.125 m in four replications. Alfalfa was grown as forecrop. A potato and wheat production area (III-C2) with a height of 375 m above the sea level. The experimental area is characterized by warm, slightly wet weather with an average annual temperature of 7.8 °C and average annual precipitations of 666 mm. Basic fertilizing was planned before the sowing in the form of 100 kg of Ammonium nitrate containing dolomite (27% N), 100 kg of 60% KCl (60% of K_2O), and100 kg of MAP (Monoammonium phosphate 12% N and 52% P_2O_5). Selenium was foliar applied in doses 25 g and 50 g Se per hectare in a solution form of sodium selenate (Na_2SeO_4). The harvest was realized by a small plot harvester in BBCH 91. The results of the experiments showed a statistically non-significant effect on microelements and most macroelements. Only sulfur content in oat grains was statistically significantly influenced by Se foliar treatment. The contents of ash, starch, and fat in oat grains were monitored, which showed statistically significant effect only in fat. Se content in grains showed a statistically significant increase by both Se foliar treatments.

Keywords: selenium; macroelements; microelements; oat grain; quality

INTRODUCTION

Selenium is one of the essential mineral elements for human nutrition **(White and Brown, 2010)**. The Se deficiency is associated with various diseases such as hypothyroidism, cardiovascular disease, a weakened immune system, male infertility, cognitive decline, and increased incidence of various cancers **(Fairweather-Tait et al., 2011)**. Selenium is included in nearly 30 selenoproteins or selenoenzymes **(Rayman, 2012)**. The level of selenium in the body depends on its concentration in the food. The selenium gets primarily to the food chain from the soils and drinking water. Its content in plants is a function of the conditions of the soil-plant system. The daily intake of Se varies geographically. Worldwide, it is estimated that over one billion people ingest Se below the recommended dose of 55 $\mu g.day^{-1}$ **(Bañuelos, Lin and Broadley, 2017)**.

Selenium is chemically similar to sulfur (S) and the absorbed selenium replaces sulfur in some proteins of plants. The enzymes are unable to distinguish between Se and S until a critical value is reached after which Se becomes toxic to a plant **(Ferri, Favero and Frasconi, 2007)**.

Macro- and microelements are significant for the growth and reproduction of plants, and their role in the human diet has been intensively investigated. Quantification and control of the mineral composition of food plants are therefore important factors for the sustainability of culture conditions which aims to increase the content of selected substances in plants **(Combs, 2011)**.

Slovak soils are generally poor in selenium, which is related to its insufficient quantity in agricultural products. Content of selenium in the crops is constantly in the spotlight of the professional public. The biological value of grown food raw materials depends on a qualitative state of growing mediums – soils. Biogenic elements presented in the soils are taken by plants and thereby entering the food chain. Plants can receive the inorganic selenium added to the soil (in the form of selenate and selenite) and convert its part or all of it into the organic components. Agronomic biofortification through the application of fertilizers enriched with selenium is one of the possible ways of its content increasing in the soil. On the other hand, there is

a potential danger of soil contamination. Due to the selenium content increasing in edible parts of a plant its combination with other biofortification approaches is promoted, such as foliar biofortification, i.e. selenium application directly to the plant **(Graham et al., 2007)**. Foliar biofortification can provide a large-scale intake of minerals with antioxidant properties for human as well as an increase of certain biologically active substances as a result of their synergies **(Hegedűsová et al., 2015)**.

Scientific hypothesis

We expect a significant effect of foliar Se application on macro-and micro-elements and selected quantitative parameters (the content of ash, starch, and fat) in oat grains.

MATERIAL AND METHODOLOGY

Small field nutritional experiments were carried out at the Research and Breeding Station Vígľaš – Pstruša in the last decade of March in the years 2014, 2015, 2016. The used variety of oat (*Avena sativa* L.) was Valentin. The experiment was realized with the soil type Luvisol Pseudogley. The experiment was performed by a block method within a parcel size of 10 m^2 (8 x 1.25 m) in four replications. The seeding rate represented 5 million of germinating grains per hectare with the span of rows amounting to 0.125 m. Alfalfa was grown as forecrop.

A potato and wheat production area (III-C2) with a height of 375 m above the sea level. An experimental area is characterized by warm, slightly wet weather with an average annual temperature of 7.8 °C and average annual precipitations of 666 mm.

The influence of Se salts foliar applied was monitored for qualitative changes in oat grains during the small field experiment. The agrochemical analysis of soil is stated in Table 1. Basic fertilizing was planned before the sowing in the form of 100 kg of Ammonium nitrate containing dolomite (27% N), 100 kg of 60% KCl (60% of K_2O), and 100 kg of MAP (Monoammonium phosphate 12% N and 52% P_2O_5). 39 kg of Nitrogen, 49.8 kg of Potassium, and 22.9 kg of Phosphorus per hectare were applied by the used fertilizers. Selenium was foliar applied in doses of 25 g and 50 g Se per hectare in the solluted form of sodium selenate (Na_2SeO_4). The supplementary fertilization was realized by hand (dew machine STIHL). The spraying dose of solutions was 400 $L.ha^{-1}$. The application was planned during a growth phase of oat heading in BBCH 53 on 06 June 2014, 12 June 2015, and 07 June 2016. The experimental variants were the following:

Variants:

K – a control without foliar biofortification,
Se25 – an application of 25 g $Se.ha^{-1}$ in the form of an aqueous solution of sodium selenate in the stage of heading,
Se50 – an application of 50 g $Se.ha^{-1}$ in the form of an aqueous solution of sodium selenate in the stage of heading.

The harvest was realized by a small plot harvester in BBCH 91 (a growth staging scale of cereals – an over-ripe phase). Effect of fertilizing variants on Se content and selected Macro- and Micro – elements and content of ash, starch, and fat in oat grains were evaluated and analyzed after the harvest. Soil and grain analyses were determined by common methods.

The samples were mineralized by a microwave decomposition method during the increased pressure with used reagents (hydrogen peroxide and concentrated nitric acid) in the following conditions:
Max. power: 800
Power: 100%
Ramp time: 20 minutes
Temperature: 170 °C
Hold 15 minutes.

The achieved mineralizate was poured into a volumetric bank and deionized water was added to the capacity of 25 mL.

Se content in wheat grains was determined by ICP-MS method (inductivelycoupled plasma mass spectrometry).

Principles of measurement:

- introduction of the measured solution into the high-frequency plasma, where the energy transfer processes from the plasma cause evaporation of the solvent, atomization, and ionization of the elements,
- extraction of ions from plasma via an interface with built-in ion optics and from the separation of ions in a mass spectrometer based on their mass to charge ratio,
- ion transfer through a mass filter (quadrupole) and from electron multiplier detection.

Equipment model: ICP-MS Agilent 7900, the country of origin is Japan.

Statistical analysis

The achieved experimental results were statistically evaluated by standard methods using the Statgraphics plus 5.1 statistical software (Rockville, USA). A multifactor ANOVA model was used for the individual treatment comparison at $p = 0.05$, with separation of the means by the *LSD* multiple-range test.

RESULTS AND DISCUSSION

Gluten-free plants and their products are the subject of interest for nutritionists, food technologists, and people with longlife gluten-free diet suffering from coeliac disease. Oat belongs to 27 grains gluten-free crops in the European Union, especially to the gluten-free cereals such as corn, buckwheat, amaranth, rice, teff, and quinoa. Popular gluten-free cererals (corn, rice, buckwheat) contain 2.8 $\mu g.100g^{-1}$ of selenium on average and in less popular crops (amaranth, teff, and quinoa) the content of selenium content is 10.8 $\mu g.100g^{-1}$ on average **(Rybicka et al., 2015)**.

The concentrations of macroelements were determined in the oat grains. In the present study, the variation in the minerals (Ca, K, Mg, and P) that are essential for good preventive nutrition of humans **(Harmankaya, Özcan and Gezgin, 2012; Rayman, 2012)** are evaluated and discussed. Metabolic pathways of sulfur and nitrogen are influenced by assimilation of selenium in plants. A recent study was focused on influence of selenium treatment on nitrogen and sulfur secondary metabolites with expected health benefits **(Malagoli et al., 2015)**.

In Table 2 the results of macroelements N, P, K, Ca, Mg are stated, showing statistically nonsignificant influence,

but the Se25 foliar application confirmed a statistically significant effect increasing of sulfur content 1.00 g.kg^{-1}. The same tendencies are confirmed by a two-year experiment with maize grains, where a statistically nonsignificant effect on macronutrients N, P, K, Ca and Mg contents were signed **(Wang, et al., 2013)**. By contrast, a two-year experiment showed the results, in which Se foliar application at 50 g Se ha^{-1} confirmed statistically significant decrease of S, K, Ca in garlic **(Põldma et. al, 2011)**. Interesting results were achieved in the maize experiments, where contents of macronutrients in aerial parts of maize depended on a selenium concentration in the nutrient solution. Selenium at concentrations of 50 and 100 μmol.dm^{-3} caused a significant increase in phosphorus and calcium. Potassium significantly increased at the presence of 25 μmol Se·dm^{-3}, while decreasing under the influence of 100 μmol Se·dm^{-3}. The presence of selenium in the medium did not have a significant influence on magnesium. An excessive concentration of selenium affected the plant growth. A lower selenium concetration 5 μmol.dm^{-3} stimulated the process of root elongation positively, but higher doses of selenium concentration 50 and 100 μmol.dm^{-3} decreased not only a root tolerance index but also dry mass accumulation. Thus, from the above mentioned, it might mean that selenium in solution at high doses causes the disturbance of plant mineral balance.

Table 1 Agrochemical soil characteristics determined before the trial establishment.

Soil analyses	2014	2015	2016
pH/KCl	6.12	5.65	6.62
Nan (mg.kg^{-1})	10.2	12.4	10.5
P Mehlich III (mg.kg^{-1})	57.5	77.5	33.8
K Mehlich III (mg.kg^{-1})	207.2	237.5	125.0
Se-total content (HF + HNO_3 + HCl) (mg.kg^{-1})	0.21	0.20	0.21

Table 2 Effect of foliar Se application on content of N, P, K, Ca, Mg and S in oat grains.

Dose Se^{6+} (g Se.ha^{-1})	Nutrient content (g.kg^{-1}), mean of years 2014, 2015, 2016					
	N	P	K	Ca	Mg	S
0	18.4 ±0.7a	3.28 ±0.23a	8.00 ±2.81a	0.57 ±0.20a	1.51 ±0.18a	0.91 ±0.06a
25	18.0 ±1.0a	3.20 ±0.37a	8.33 ±3.00a	0.55 ±0.18a	1.47 ±0.10a	1.00 ±0.02b
50	17.8 ±0.4a	3.06 ±0.33a	8.00 ±2.81a	0.53 ±0.14a	1.53 ±0.16a	0.93 ±0.08a
$LSD_{0,05}$	0.6	0.26	2.39	0.14	0.12	0.05

Note: 0 – 0.0 g Se.ha^{-1}; 25 – 25 g Se.ha^{-1}; 50 – 50 g Se.ha^{-1}; the values in the columns with different letters are significantly different from each other at p <0.05.

Table 3 Influence of foliar Se application on content of Cu, Fe, Mn, Zn in oat grains.

Dose Se^{6+} (g Se.ha^{-1})	Nutrient content (mg.kg-1), mean of years 2014, 2015, 2016			
	Cu	Fe	Mn	Zn
0	5.39 ±0.85a	95.2 ±24.7a	42.4 ±7.3a	58.3 ±10.5a
25	4.99 ±1.39a	98.0 ±31.6a	40.7 ±9.0a	58.0 ±10.8a
50	5.38 ±0.91a	129.0 ±67.1a	41.7 ±10.4a	54.3 ±7.0a
$LSD_{0,05}$	0.89	37.5	7.5	8.0

Note: 0 – 0.0 g Se.ha^{-1}; 25 – 25 g Se.ha^{-1}; 50 – 50 g Se.ha^{-1}; the values in the columns with different letters are significantly different from each other at p <0.05.

Table 4 Influence of Se foliar application on content of ash, starch and fat in oat grains.

Dose Se^{6+} (g Se.ha^{-1})	Content (%), mean of years 2014, 2015, 2016		
	ash	starch	fat
0	3.98 ±1.13a	39.4 ±1.7a	3.72 ±0.26ab
25	4.10 ±1.48a	39,1 ±1.7a	3.58 ±0.39a
50	4.13 ±0.94a	39.9 ±2.4a	4.01 ±0.43b
$LSD_{0,05}$	1.0	1.6	0.31

Note: 0 – 0.0 g Se.ha^{-1}; 25 – 25 g Se.ha^{-1}; 50 – 50 g Se.ha^{-1}; the values in the columns with different letters are significantly different from each other at p <0.05.

Table 5 Effect of foliar Se application on Se content in oat grains.

Dose Se^{6+} (g Se.ha^{-1})	Se content (mg.kg^{-1})			
	2014	2015	2016	Average of years
0	<0.03a	<0.03a	<0.03a	<0.03a
25	0.35 ±0.02b	0.13 ±0.05b	0.36 ±0.08b	0.28 ±0.12b
50	0.56 ±0.11c	0.25 ±0.06c	0.53 ±0.07c	0.45 ±0.16c
$LSD_{0,05}$	0.13	0.08	0.12	0.12

Note: 0 – 0.0 g Se.ha^{-1}; 25 – 25 g Se.ha^{-1}; 50 – 50 g Se.ha^{-1}; the values in the columns with different letters are significantly different from each other at p <0.05.

The result is the increase of high amount of calcium and phosphorus in root shoot tissue **(Hawrylak-Nowak, 2008)**.

Selenium foliar application showed a statistically non-significant effect on the content of microelements Cu, Fe, Mn, and Zn in oat grains (Table 3). Three-year average values of microelement contents after Se foliar application were Cu 4.99, 5.38 $mg.kg^{-1}$, Fe 98.0, 129.0 $mg.kg^{-1}$, Mn 40.7, 41.7 $mg.kg^{-1}$ and Zn 58.0, 54.3 $mg.kg^{-1}$. In experiments with maize grains according to **Wang et al. (2013)** Se soil and foliar applications did not confirm statistically significant influence on monitored microelements (Fe, Mn, Cu, Zn).

Se is a microelement influencing a proper function of organism, but with a negative effect in dosage. It means that selenium disposes with a very interesting property, where the main sign is a very thin line between dietary deficiency and toxicity **(Kieliszek and Błażejak, 2013)**. Recent experiments have been focused on tracking the selenium content in selected crops intented for human consumption. A consumption of selenium affected products becomes the main source of selenium in human nutrition **(Chen et al., 2002; Hawkesford and Zhao 2007; Lyons, Stangoulis and Graham, 2004; Ramos et al., 2010; Schiavon et al., 2013)**.

Qualitative parameters in oat grains (ash, starch, and fat) after foliar applications were studied. The achieved values of these parameters are ash 4.10, 4.13%, starch 39.1, 39.9%, and fat 3.58, 4.01%. A statistically significant influence on selected qualitative parameters was achieved only between foliar Se applications. The statistically non-significant effect was shown between the control variant (without Se treatment) and variants with Se foliar treatment (Table 4). Similar results were described in the experiment according to **Havrlentová et al. (2013)**, where the qualitative parameter was β-D-glucan. A statistically significant difference was found between 2 variants of 5 by hulled oat. The application of fertilizers with selenium at naked oat grains was statistically non-significant. Other experiments with tomatoes showed interesting results in qualitative parameters, where foliar application of selenium had a positive effect on the increase of total polyphenol. The influence of Se biofortification on the content of vitamin C and carotenoids was not detected. Selenium foliar fertilization in dosage 150 $g.ha^{-1}$ is suitable way of tomato fruits enriching in polyphenols, without the negative effect on other antioxidants content **(Andrejiová et al., 2019)**.

Foliar application of Se is usually more efficient than soil application whenever the element is prone to be sorbed into soil particles **(Arthur, 2003)**, which is the case of Oxifact, Oxisols have positively charged surfaces that retain many anions **(Lopes and Guimarães Guilherme, 2016)**, including selenite and selenate, thus decreasing the availability of soil apsoil-appliedis interaction is especially strong for selenite, when compared with selenate. Irrespective of the form, in both cases (i.e., Se selenite or Se-selenate), since soil particles might absorb Se, the foliar application requires smaller doses and are more efficient **(Fernandes Boldrin et al., 2013; de Oliveira et al., 2018; Zhu et al., 2009)**. Se, best acceptable in the form of Na_2Se0_4 **(Eurola et al., 1990)**, is primarily transported into the chloroplast. SeO_4^{-2} then activates ATP-sulphurylase and forms of APSe by being reduced to selenite. This results in the production of amino acids, such as selenocysteine and selenomethionine. Selenium increases the content of amino acids, particularly isoleucine **(Duma and Karklina, 2008)**. Selenomethionine can be methylated to dimethylselenid through evaporation in the plant. It is particularly difficult and not easy to determine the levels of Se in the plant **(Brown and Shrift, 1982)**, as well as determine whether Se is essential for plant microelements. However, there is evidence that a higher content of Se (depending on the concentration of sulphur) has a positive impact, not only on levels of amino acids but also on plant growth and multiplication **(Ferri, Favero and Frasconi, 2007; Mora et al., 2015; Pennanen, Xue and Hartikainen, 2002; White and Broadley, 2009; White et al., 2007)**.

As expected, the Se content showed a statistically significant increase in oat grain after Se foliar treatment, as it is confirmed in Table 5. The average of years 2014 – 2016 in Se treatments were 0.28 ±0.12 $mg.kg^{-1}$ and 0.45 ±0.16 $mg.kg^{-1}$. Our achieved results of contents are comparable to multiple experiments **(Aspila, 2005; Galinha et al., 2012; Ventura, 2008)** with a positive effect on Se content in crops and vegetables after different application doses of selenium **(Galinha et al., 2012; Ventura, 2008)**.

Selenium is an essential mineral element for the well-being of animals and a beneficial element for plants. However, excess Se can be toxic to both animals and plants. There is considerable interest in understanding how plants acquire and accumulate Se, not only to facilitate appropriate dietary Se intakes for animal and humans, which often requires Se biofortification of edible crops but also to remediate the land contaminated anthropogenically by an excess of Se and to appreciate the ecology of native plants inhabiting seleniferous soils.

CONCLUSION

This study monitored the effect of Se foliar application on macro- and microelements and selected qualitative parameters in oat grains. In most cases a statistically nonsignificant effect of Se treatment on macroelements and microelements content in oat grains was found. The only sulfur amount increased 1.00 ±0.02 by foliar Se application in sodium selenate form in a dose of 25 g $Se.ha^{-1}$.

Qualitative parameters (ash, strarch, and fat) showed a statistically significant effect in fat content on both variants with Se treatment. Se contents in oat grains proved a statistically significant increase in both foliar Se applications, where the highest amount of Se 0.45 ±0.16was achieved on variants treated in dose of 50 g Se ha^{-1}.

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ASSESSMENT OF A NEW ARTIFICIAL BUCKWHEAT SPECIES *FAGOPYRUM HYBRIDUM* AS A SOURCE OF PLANT RAW MATERIALS COMPARED TO *F. TATARICUM* AND *F. ESCULENTUM*

Elena Klimova, Ivan Fesenko, Elena Kuznetsova, Ján Brindza, Gyunesh Nasrullaeva, Olga Rezunova, Elena Kuznetsova

ABSTRACT

A promising way to increase the use of buckwheat is the wider introduction of technologies for its processing, including grinding of non-hulled grain. It requires the search for new plant materials with more suitable characteristics. In this work, the possibilities to use the grain of a new artificial buckwheat species *Fagopyrum hybridum* for flour production are studied in comparison with two cultivated species *F. tataricum* and *F. esculentum*. Some chemical characteristics of *F. hybridum* flour were evaluated. According to the size of the kernel fragments in different modes of milling within each species the significant differences were identified within *F. esculentum* and *F. hybridum* (p <0.001 and p <0.05, respectively); there were no significant differences within *F. tataricum* (p >0.1). Fragments of the seed hulls of *F. tataricum* and *F. hybridum* compared to ones of *F. esculentum* were distinguished by the absence of pronounced acute angles. For the cultivated species, amino acid compositions of grain protein of the studied samples manifest no strong deviations from earlier published results. The new species *F. hybridum* has the amino acid composition similar to ones of the both cultivated species with slight superiority in the content of all essential amino acids. So, the content of Cysteine, Tryptophan, Arginine, Lysine, Methionine, Leucine + Isoleucine, Threonine, Histidine and Valine in seeds of *F. hybridum* was 5.2, 15.0, 25.8, 30.2, 31.2, 36.0, 38.4, 41.1 and 46.2% higher compared to *F. tataricum* and 11.1, 43.7, 39.2, 3.7, 31.2, 15.2, 14.8, 20.0, 18.9% higher compared to *F. esculentum*. Using DPPH it was assessed the antioxidant activity (AOA) of whole grain flour of three buckwheat species and decreasing of the AOA during heating up to 100 °C. After water extraction the AOA was maximal for *F. tataricum* flour; *F. hybridum* and *F. esculentum* manifested similar values with the same decline dynamics during heating. After ethanol extraction the flour of *F. hybridum* shown higher AOA compared to both cultivated species before temperature treatment (1.3 times) as well as after heating to 100 °C (1.2 times). The results of the analysis of the fractional composition of flour from the whole grain of the three buckwheats shown the fragments of the seed hulls of *F. tataricum* and *F. hybridum* compared to ones of *F. esculentum* were characterized by the absence of pronounced acute angles. Additional experiments are needed to optimize the technology of whole-grain buckwheat flour. But the grain of *F. tataricum* and *F. hybridum* looks like more suitable for these purposes than the non-hulled grain of *F. esculentum.*

Keywords: buckwheat; grain; flour; food industry

INTRODUCTION

Buckwheat, a pseudo-cereal crop belonging to the family Polygonaceae, genus *Fagopyrum*, is a popular health food in Asian and European countries **(Kreft et al., 2003)**. Together with well-balanced chemical composition **(Bonafaccia, Marocchini and Kreft, 2003; Zhu, 2016)** including the optimal amino acid composition of seed storage protein **(Prakash et al., 1987; Jiang et al., 2007)** buckwheat manifests a high level of antioxidant activity due to the content of flavonoids **(Holasova et al., 2002)**. In buckwheat grain it was identified rutin, quercetin, and flavone C-glycosides **(Zielińska et al., 2012)**. It has several pharmacological functions such as anti-inflammatory, anti-diabetic **(Lee et al., 2016**; **Kamalakkannan and Prince, 2006)**, blood capillary strengthening properties **(Chua, 2013)**, and lipid-lowering activity **(Tomotake et al., 2015)**. Also, rutin has cardioprotective effects **(He et al., 1995**; **Wojcicki et al., 1995**; **Annapurna et al., 2009)**. It allows considering buckwheat as a functional food. Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) contains approximately 100-fold higher amounts of rutin in its seeds compared to common buckwheat **(Fabjan et al., 2003)**. Sometimes buckwheat flour is used for the improvement of wheat-based products. So, wheat bread with buckwheat flour shown the level of antioxidant activity depends on the percentage of buckwheat flour, and the rutin content in such bread ranged from 7.76 to 26.90 mg kg^{-1} **(Lin et al., 2009**; **Bojňanská et al., 2009**; **Brindzová et al., 2009)**. Such products are recommended especially for people who

live in conditions of oxidative stress **(Kuznetsova et al., 2018)**.

In Russia, at present, the traditions of buckwheat consumption mainly as a groats crop promote cultivating only common buckwheat which grain is more suitable for groats production. In China and several other Asian countries where buckwheat grain is used mainly for the production of flour the Tartary buckwheat is also cultivated. Grinding of non-hulled grain is one of most perspective approach for use of buckwheat since it is a simple method which allows producing various types of flour for making noodles, pasta, bread, confectionery, etc. **(Steadman et al., 2001)**. Grinding grain with hulls reduces the relative content of nutrients in the flour obtained but increases the content of dietary fiber **(Dziedzic et al., 2012)**. Buckwheat grains can be milled using any equipment designed for grinding cereals, for example, the millstones or roller mill **(Mazza and Oomah, 2005)**. Millstones are more often used to produce whole grain flour for making pancakes at home. White flour can be obtained from such flour by removing the bran by sieving. The fineness of grinding with millstones can be different and is adjusted by changing the gap between them. Grinding with millstones is a one-step process, unlike grinding with a roller mill, where the process can be divided into several stages with the release of several flour fractions **(Ohinata et al., 2001)**.

It seems promising to search, create, and evaluate new buckwheat samples which could be more suitable raw materials for the development of deeper processing products. Using hybridization *F. tataricum* (4x = 32) × *F. giganteum* together with selection in late generations (F_{10} and later) recently was created a new buckwheat species *F. hybridum* **(Fesenko and Fesenko, 2010)**. The artificial species manifests competitive yield ability and may be considered for registration as a cultivar **(Fesenko et al., 2017)**.

An objective of this paper was the evaluation of *F. hybridum* grain properties, including amino acid composition, antioxidant activity, and characteristics of fragments of kernels and hulls after milling by both roller mill and millstones, compared to ones of *F. tataricum* and *F. esculentum*.

Scientific hypothesis

The whole grain of *F. hybridum* (a new species obtained using hybridization of tetraploid *F. tataricum* with artificial amphidiploid *F. giganteum* Krotov) and *F. tataricum* (Tartary buckwheat) is more perspective row material for the production of functional food in terms of deep processing technology compared to a whole grain of *F. esculentum* (common buckwheat) which usually used for groats production.

MATERIAL AND METHODOLOGY

The work involved one sample of each species. *F. tataricum* – an accession k-17 from the Federal Research Center N.I. Vavilov All-Russian Institute of Plant Genetic Resourses (VIR) collection (St.Petersburg); *F. hybridum* is a new species of hybrid origin which has been created at the Federal Research Center of Grain Legumes and Groats Crops, Orel, Russia **(Fesenko and Fesenko, 2010)**; *F. esculentum* was represented by cv. Devyatka (Federal Research Center of grain legumes and groats crops).

The grinding of the grain was carried out both on a millstone with gaps of 0.01 and 0.3 mm, and on roller mill with two frequencies of rotation, $6.5x10^3$ and $10x10^3$ turns per minute. Micrographs of the grains fragments were taken with an AxioCam MRc5 camera (Axio Imager microscope. Al, Carl Zeiss). The measurements were made using the AxioVision program.

Antioxidant activity (AOA) was measured using spectrophotometry in both alcohol and water extracts based on inhibition of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical **(Silva et al., 2005)**. Flavonoids were extracted with both 96% ethanol and water. 1g of milled grain was mixed with 25 mL of ethanol or water. Extraction was carried out for 24 hours with constant stirring. The extracts were filtered. 0.025 DPPH was dissolved in 100 mL of 96% ethanol. 10 mL of the solution was mixed with 90 mL of 96% ethanol. The optical density of the solution was measured after 30 minutes on a spectrophotometer in a cuvette with a thickness of 1 cm, at a wavelength of 515 nm. To determine the antioxidant activity of the studied extract, 0.1 mL of the filtrate was added to 3.9 mL of the solution of DPPH, mixed, and placed in a dark for 5 – 10 minutes. Then the optical density of the solution was measured using a spectrophotometer at a wavelength of 515 nm.

To determine the thermal stability of bioflavonoids, the extracts were gradually heated in a water bath (t = 20 – 100 ºC), and AOA was measured at 20, 40, 60, 80, and 100 ºC. The antioxidant activity (AOA) of the extracts is calculated by the formula $AOA = \frac{A1-A2}{A1} \times 100\%$, where A1 is the optical density of the DPPH solution before adding the investigated extract; A2 – optical density of the DPPH solution after adding the investigated extract.

The amino acid composition was evaluated using amino acid analyzer BIOCHROM (Biochrom Ltd., Great Britain).

Statistical analysis

Standard statistical analysis was conducted using MS Excell in combination with XLSTAT. The significance of differences between compared variants was analyzed using t-statistics (a two-sample *t*-test for independent samples); p-value is indicated where it is necessary.

RESULTS AND DISCUSSION

Results of different milling approaches

Millstones milling

The results of coarse and fine grinding were not entirely unambiguous. By the size of the hulls fragments, the maximum values were significantly higher for coarse grinding, but significant differences between the mean values were only for *F. hybridum* (t = 3.20; p = 0.02) (Table 1). Also, in the case of *F. tataricum*, the mean value of this trait was higher for fine grinding (albeit the difference was not significant). The maximal fragments of the kernel were larger in all cases on coarse grinding, but the average values were significantly higher only for *F. tataricum* (t = 6.14; p = 0.001) and *F. hybridum* (t = 2.04; p = 0.05).

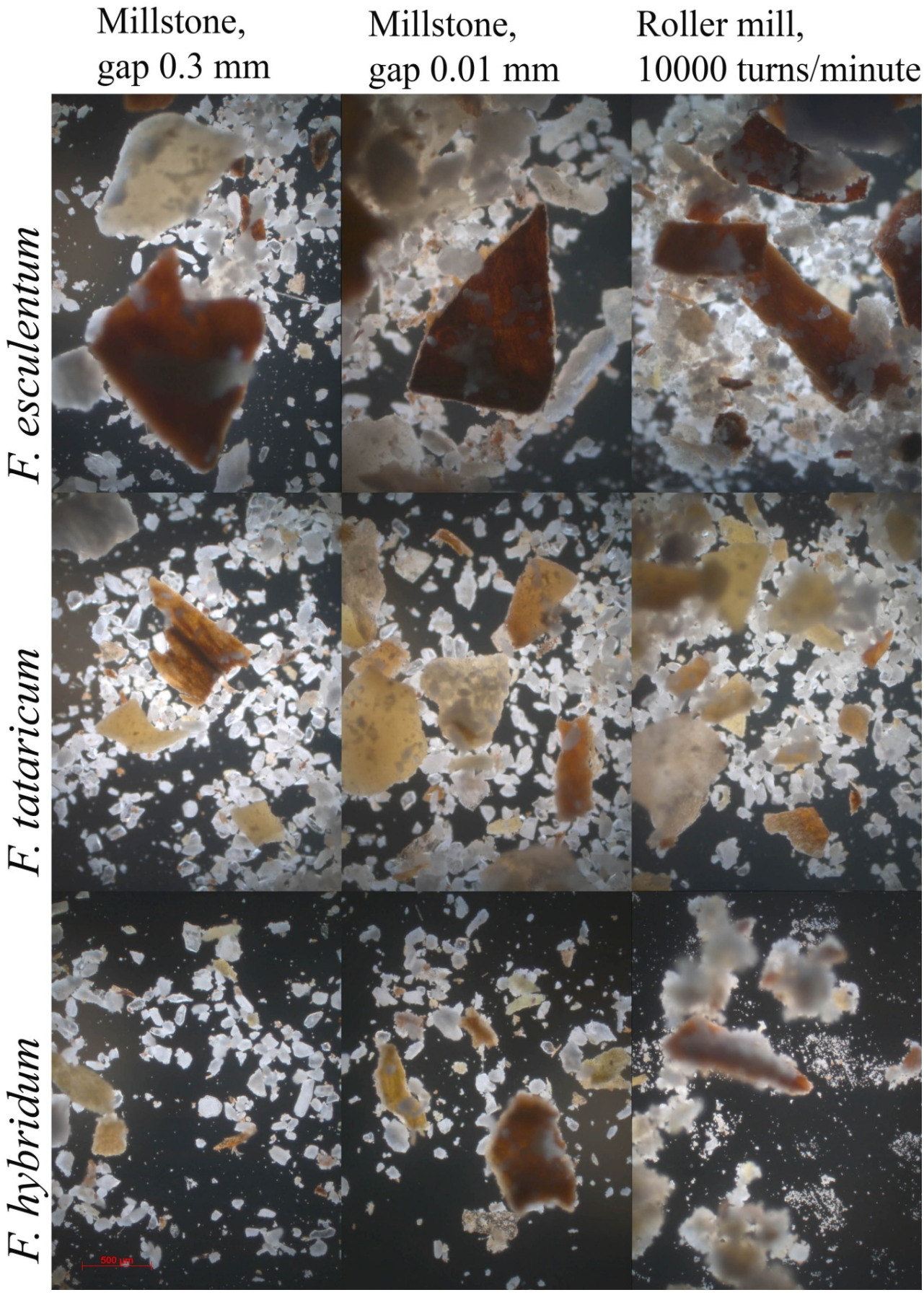

Figure 1 Kernels and hulls fragments of three buckwheat species after millstone (with gaps 0.3 and 0.01 mm) and rolling (10000 turns per minute) milling.

Table 1 Sizes (μm) of the kernels and hulls fragments of three buckwheat species after millstone milling.

Species	Fragments of	Gap = 0.01 mm		Gap = 0.3 mm	
		X ±m	Range	X ±m	Range
F. tataricum	**kernel**	66.1 ±1.7	21.4 – 158.9	90.0 ±3.5	46.7 – 298.8
	hulls	255.2 ±32.2	44.1 – 1429.0	207.7 ±33.1	14.6 – 2104.5
F. hybridum	**kernel**	90.9 ±4.1	27.4 – 641.7	108.5 ±7.6	46.2 – 851.0
	hulls	136.5 ±14.9	17.6 –675.9	207.2 ±16.3	20.7 – 1657.5
F. esculentum	**kernel**	128.6 ±7.6	29.6 – 936.9	96.0 ±7.1	24.3 – 1095.2
	hulls	271.9 ±78.2	22.4 – 1927.0	317.6 ±92.5	66.6 – 2527.0

Table 2 Sizes(μm) of the kernels and hulls fragments of three buckwheat species after milling by roller mill.

Species	Fragments of	Rotation frequency, turns per minute			
		$6.5x10^3$		$10x10^3$	
		X ±m	Range	X ±m	Range
F. tataricum	**kernel**	65.5 ±1.7	25.5 – 117.0	62.1 ±2.2	28.4 – 137.5
	hulls	172.8 ±11.3	41.9 – 625.5	233.9 ±55.3	48.3 – 1755.2
F. hybridum	**kernel**	70.1 ±4.6	32.3 – 154.7	59.0 ±3.1	13.2 – 208.6
	hulls	154.3 ±13.1	17.6 – 1066.8	116.6 ±16,3	10.7 – 557.7
F. esculentum	**kernel**	60.2 ±1.8	25.5 – 119.5	50.0 ±1.7	13.1 – 108.9
	hulls	347.8 ±84.4	43.2 – 1540.9	387.5 ±92.5	71.8 – 1397.1

In the case of *F. esculentum*, the fragments othe kernels were on average significantly larger on fine grinding (t = 3.13; p = 0.01). When comparing species on coarse grinding, in the size of the flour significant differences were found only between *F. tataricum* and *F. hybridum* (t = 2.21; p = 0.05). On fine grinding, no significant differences were found in the size of the hulls fragments between *F. esculentum* and the other two species, on average, although the maximal values were higher for *F. esculentum*. Fragments of the hulls of *F. tataricum* were significantly larger compared to *F. hybridum*. The fragments of the *F. tataricum* kernel were significantly smaller than in the other two species (p = 0.001). Fragments of the kernel of *F. esculentum* were larger than those of *F. hybridum* (t = 4.37; p = 0.001).

Roller milling

Grinding on a roller mill was carried out in two modes (Table 2). According to the size of the kernel fragments in different modes of milling within each species, the significant differences were identified within *F. esculentum* and *F. hybridum* (p <0.001 and p <0.05, respectively); there were no significant differences within *F. tataricum* (p >0.1). There were no significant differences in the size of hulls fragments in any case.

There were significant differences between *F. esculentum* and two other species in the size of the kernel fragments in all cases (the fragments of the *F. esculentum* kernels are smaller). The fragments of the *F. esculentum* hulls were noticeably larger than those of the other two species: only in comparison with *F. tataricum* when grinding at 10,000 turns per minute the differences were not significant. The differences between *F. tataricum* and *F. hybridum* in the size of the fragments of both the kernels and hulls were significant only when grinding at 10,000 turns.

It should be noted that the fragments of the seed hulls of *F. tataricum* and *F. hybridum* compared to ones of *F. esculentum* were distinguished by the absence of pronounced acute angles (Figure 1). Additional experiments are needed to optimize the technology of whole-grain buckwheat flour. But the grain of *F. tataricum* and *F. hybridum* looks like more suitable for these purposes than the non-hulled grain of *F. esculentum*.

Antioxidant activity (AOA) of flour and decline dynamics of the AOA during heating

Although some antioxidant activity (AOA) is characteristic of many plants **(Bandyukova and Sergeeva, 1974**; **Chua, 2013)**, including some wheat species **(Kuznetsova et al., 2018; Kuznetsova et al., 2019)**, buckwheat, especially Tartary buckwheat grain contains an outstanding amount of antioxidants, and it is one of the main advantages of the crop **(Kitabayashi et al., 1995a; Kitabayashi et al., 1995b**; **Ohsawa and Tsutsumi, 1995; Kreft et al., 1999**; **Holasova et al., 2002; Fabjan et al., 2003; Jiang et al., 2007; Zielińska et al., 2012; Kreft, 2016; Lee et al., 2016)**. The processing of grain into bread and confectionery products is usually associated with heat treatment at some stages; therefore it is necessary to evaluate the resistance of antioxidants contained in flour to heat. Using DPPH it was assessed the AOA of flour from whole grain of three buckwheat species and decreasing of the AOA during heating up to 100 °C. After water extraction the AOA was maximal for *F. tataricum* flour; *F. hybridum* and *F. esculentum* manifested similar values with the same decline dynamics during heating (Table 3). After ethanol extraction, the flour of *F. hybridum* shown higher AOA compared to both cultivated species before temperature treatment (1.3 times) as well as after heating to 100 °C (1.2 times). Since alcohol extracts antioxidants more efficiently compared water, the results of alcohol extraction reflect the ratios of their contents in different types of flour. The method used does not give an accurate estimate of the ratio of flavonoids in seeds of different species.

Table 3 Dynamics of antioxidant activity (AOA) of buckwheat flour extracts during heating.

Species	Flavonoid extraction with	Antioxidant activity (% inhibition of DPPH) after temperature treatment at				
		20 °C	40 °C	60 °C	80 °C	100 °C
F. esculentum	water	6.0 – 6.1	5.0 – 5.8	2.8 – 2.9	1.9 – 2.5	1.3 – 2.0
	ethanol	23.6 – 24.8	16.2 – 20.2	14.9 – 16.1	14.9 – 15.0	13.5 – 13.7
F. tataricum	water	29.6 – 30.6	25.5 – 26.0	22.5 – 23.5	19.8 – 20.8	17.9 – 18.4
	ethanol	41.7 – 42.8	34.2 – 34.5	29.2 – 30.1	28.4 – 28.8	26.8 – 27.2
F. hybridum	water	9.4 – 9.6	5.0 – 5.3	4.4 – 4.7	4.0 – 4.3	3.9 – 4.1
	ethanol	54.9 – 55.3	49.6 – 50.1	39.9 – 40.3	35.4 – 36.1	32.0 – 33.0

Table 4 Amino acid composition of *Fagopyrum* sp. (g.100g^{-1} flour).

Amino acid	*F.hybridum*	*F.tataricum*	*F.esculentum*
Arginine	0.78	0.62	0.56
Lisyne	0.56	0.43	0.54
Tyrosine	0.22	0.15	0.19
Phenylalanine	0.52	0.38	0.47
Histidine	0.24	0.17	0.20
Leucine+Isoleucine	0.83	0.61	0.72
Methionine	0.21	0.16	0.16
Valine	0.44	0.30	0.37
Proline	0.52	0.34	0.41
Threonine	0.54	0.39	0.47
Serine	0.55	0.44	0.47
Alanine	0.62	0.45	0.55
Glycine	0.52	0.37	0.46
Cysteine	0.20	0.19	0.18
Glutamicacid	2.26	1.67	2.02
Asparticacid	1.17	0.84	1.03
Tryptophan	0.23	0.20	0.16

It is known that differences in flavonoid content in seeds between *F. tataricum* and *F. esculentum* can be a hundredfold. However, these results correctly rank the test samples. *F. hybridum* in total AOA exceeds *F. esculentum* 2.2 – 2.3 times and *F. tataricum* 1.3 times.

The results of water extraction reflect the availability of antioxidants for enzymes that destroy them when using flour to make a dough **(Yasuda and Nakagawa, 1994; Suzuki et al., 2002, Suzuki et al., 2004, Suzuki et al., 2014)**. For *F. tataricum*, the maximum values of the efficiency of aqueous extraction were obtained: the AOA of an aqueous solution was 71% of the AOA of an alcohol solution. For *F. esculentum*, AOA of the aqueous solution was 25% of AOA alcohol. For *F. hybridum*, the AOA of the aqueous extract was only 17% of the AOA of the alcohol extract. Since the AOA of the alcohol extract of *F. hybridum* was maximal, this species is probably the most promising raw material for the production of products with high AOA. Lower AOA of aqueous extract of *F. hybridum* compared to *F. tataricum* can be elucidated either higher activity of antioxidants degradation enzymes or lower solubility of the antioxidants in water.

Amino acid composition

In spite of significant differences between the cultivated buckwheats, *F. esculentum* and *F. tataricum*, for the SDS PAGE spectra of seed storage proteins **(Rogl and Javornik, 1996; Lazareva and Fesenko, 2007; Lazareva et al., 2007; Li et al.,2008)**, both the species have a well-balanced amino acid composition of seed protein, with some variation among both species **(Prakash et al., 1987; Yang and Lu, 1992; Bonafaccia et al., 1994)**. *F. hybridum* has not been previously studied in this regard. We analyzed it for accessions studied in the work. The results are presented in Table 4.

The results manifest no strong deviations from earlier published results. The studied accession of new species *F. hybridum* has amino acid composition of seed protein similar to one of the cultivated species. In terms of the content of all essential amino acids, the sample *F. hybridum* is at least slightly superior to the samples *F. esculentum* and *F. tataricum* studied in our work. So, content of Cysteine, Tryptophan, Arginine, Lisyne, Methionine, Leucine + Isoleucine, Threonine, Histidine, and Valine in seeds of *F. hybridum* was 5.2, 15.0, 25.8, 30.2, 31.2, 36.0, 38.4, 41.1 and 46.2% higher compared to *F. tataricum* and 11.1, 43.7, 39.2, 3.7, 31.2, 15.2, 14.8, 20.0, 18.9% higher compared to *F. esculentum*.

CONCLUSION

So, the new species *F. hybridum* is better in some biochemical characteristics in comparison to cultivated buckwheats, *F. esculentum,* and *F. tataricum*. The total antioxidant activity of ethanol extract from *F. hybridum* flour was higher even compared to *F. tataricum*. Water extract from *F. hybridum* flour manifested only 17% AOA of ethanol extract. Probably, minimal efficiency of water extraction may indicate protection from dissolution by water and, accordingly, from the destruction of the flavonoids by enzymes, but the alternative explanation

about the higher activity of antioxidants degradation enzymes is not yet rejected. In terms of the content of all essential amino acids, the studied sample of *F. hybridum* exceeds the studied sample of *F. tataricum* by 5.2% (Cysteine) – 46.2% (Valine), and the studied sample of *F. esculentum* by 3.7% (Lisyne) – 39.2% (Arginine). The milling fragments of *F. hybridum* seeds hulls, as well as *F. tataricum* ones, have no pronounced acute angles, probably due to less compact structure of the hulls compared to *F. esculentum*. The non-hulled grain of both *F. hybridum* and *F. tataricum* is more suitable for production of whole-grain flour than the non-hulled grain of *F. esculentum*. Using the whole grain flour allows making the products with a high share of dietary fibers.

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IMPROVING THE QUALITY OF MILK DISPERSION IN A COUNTER-JET HOMOGENIZER

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ABSTRACT

Homogenization is a necessary process in the production of drinking milk and most dairy products. The specific energy consumption of the most common valve homogenizers reaches 8 kW $h.t^{-1}$. A promising way to reduce it is the introduction of more effective counter-jet homogenizers. The purpose of these studies is to increase the efficiency of machines of this type through fuller use of their kinetic energy. To achieve this, the design of a ring reflector was developed and experimental studies were carried out to determine its influence on the efficiency of milk fat dispersion in a counter-jet homogenizer. Calculations were made to determine the reflector's design parameters. An installation for experimental research has been developed, in which the required milk pressure is created with the help of compressed carbon dioxide. The dispersive indices of the milk emulsion were determined by computer analysis of milk sample micrographs obtained with an optical microscope and a digital camera using Microsoft Office Excel and Microsoft Visual Studio C# software using the OpenCV Sharp library. As a result of research, the formula for defining the angle of the reflector top has been determined analytically. Experimental studies proved its validity and allowed determination of the optimal diameter. A comparison of the dependence of the degree of homogenization on the excess pressure in a counter-jet homogenizer proves a 15 – 20% increase in the degree of dispersion when using a reflector. At the same time, specific energy consumption does not increase. Comparison of the distribution curves of milk fat globules by size after counter-jet homogenization and homogenization with a reflector suggests that the average diameter of fat globules for the experimental method decreases from 0.99 to 0.83 μm. This indicates the high quality of the dispersal characteristics of the milk emulsion after processing in a counter-jet homogenizer with a reflector.

Keywords: milk; homogenization; homogenizer; counter-jet homogenizers; reflector; degree of dispersion

INTRODUCTION

Homogenization is a necessary process in the production of drinking milk and most dairy products. The benefits of homogenized products are undeniable: reduced cream sludge, increased milk shelf life, improved taste and sensory properties of dairy products, increased digestion of milk fat and its even distribution throughout the product, etc. **(Dhankhar, 2014)**.

When milk is homogenized, its fat phase is dispersed (fat globules are crushed), as a result of which the average fat particle size decreases from 3 – 5 to 0.7 – 1 μm **(Walstra, Wouers and Geurts, 2006)**. This result can be achieved by exposing milk to pressure and velocity via ultrasonic, cavitation, vacuum and high-frequency electrical treatment **(Nuzhin and Gladushnyak, 2007; Samoichuk et al., 2016)**. Taking into account such a wide range of effects on the milk emulsion, dozens of types of homogenizers have been developed, which differ significantly from each other both in their design and principle of action **(Dhankhar, 2014; Fialkova, 2006)**.

A modern homogenizer must have a high efficiency (degree) of homogenization at low energy consumption. Moreover, the high degree of homogenization is crucial, which is confirmed by the fact that the vast majority of homogenizers in processing plants are valvular. When processing milk in such machines, the average diameter of fat globules is 0.75 μm, and the amount of energy consumed per unit of processed product is the highest among all existing homogenizers **(Rayner and Dejmek, 2015)**. Vacuum, ultrasonic, cavitation, mixing, electrohydraulic, screw, and spunbond devices for homogenization with significantly lower energy consumption have a lower degree of homogenization **(Dhankhar, 2014; Fialkova, 2006; Nuzhin and Gladushnyak, 2007)**. The degree of homogenization is close to that of valve rotor-pulsation and vortex

homogenizers. But a product that has been processed in a rotary homogenizer has a large fat content, which negatively affects the quality of dairy products made from such milk **(Fialkova, 2006)**.

Two types of high-efficiency jet homogenizers are distinguished, namely: jet, with a separate supply of fat phases **(Samoichuk et al., 2020)**, and counter-jet **(Samoichuk, 2008)**. Such homogenizers, the only ones among existing types, provide the highest speed of flow of the fat globule through the flow of milk plasma. After all, the Weber criterion depends on this value, which is a generalizing indicator for the main factors in the dispersion of the fat phase of milk **(Samoichuk et al., 2019)**. Weber's destruction coincides with the theories of homogenization by N. Baranovsky, P. Rebinder, A. Wittig and M. Oreshina, and by Innings' experimental studies **(Innings and Trägårdh, 2005; Oreshina, 2010)**.

A counter-jet homogenizer is not inferior to the valve type regarding the degree of dispersion of milk but has a specific energy consumption 4 – 5 times lower **(Samoichuk, 2008)**. This indicates a high potential for hydrodynamic dispersion, which is carried out when jets of milk emulsion collide.

In a study on such a homogenizer, it was found that the disruption of milk fat globules mainly occurs in the central part of the jet collision zone **(Samoichuk, 2008)**. After that, the jets divaricate in a fan shape (circularly). Their speed, and hence kinetic energy, is quite high. But this energy is not used for dispersion, so it reduces the efficiency of the homogenizer.

It is known that the disruption of milk fat globules occurs when a jet of milk collides with a hard surface **(Deynichenko et al., 2018; Nuzhin and Gladushnyak, 2007)**. Concerning counter-jet homogenizers, such a surface may be an annular reflector located in the path of milk flow after the collision.

Scientific hypothesis

This study hypothesizes that it is possible to increase the dispersion efficiency of a counter-jet homogenizer by installing an annular reflector.

This article aims to evaluate the effectiveness of dispersing milk in a counter-jet homogenizer with a reflector.

To achieve this aim, it is necessary to:

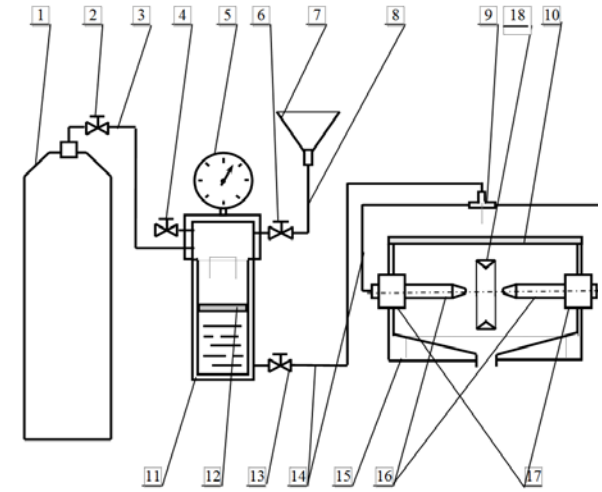
- develop the design of the annular reflector;
- experimentally determine the effect of the main parameters of the counter-jet homogenizer with a reflector on the degree of homogenization of milk;
- evaluate the dispersal indicators of the milk emulsion after homogenization.

MATERIAL AND METHODOLOGY

Experimental equipment

For experimental research, the reflector device was designed, the scheme of which is presented in Figure 1 **(Samoichuk, 2008)**.

The device consists of a chamber 15, in which the nozzles 16 are installed. The bottom of the chamber is conical with a slope to the centre, where the hole for removing milk after homogenization is placed. At the top, the chamber is closed with a transparent lid 10 to allow the process to be monitored. The sleeves 17 for adjusting the position of the nozzles with radially located screws allow the distance between nozzles to be changed and their alignment. Nozzles are collapsible and can be replaced.



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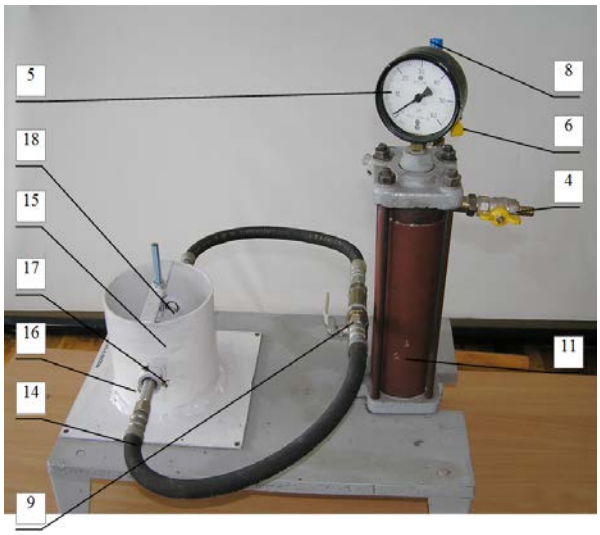

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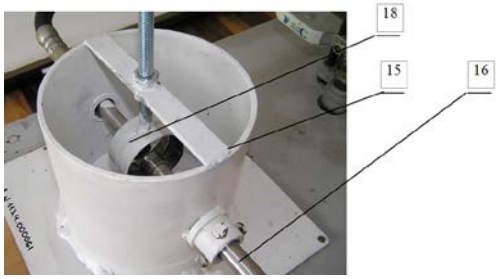


c)

Figure 1 Scheme a), general view b) and chamber c) of the laboratory device for the study of counter-jet homogenization of milk: 1 – gas cylinder; 2 – discharge valve; 3 – air duct; 4 – outlet valve; 5 – manometer; 6 – filling valve; 7 – funnel; 8 – hose; 9 – tee; 10 – cover; 11 – cylinder; 12 – piston; 13 – main valve; 14 – hydraulic hoses; 15 – camera; 16 – nozzles; 17 – bushings for adjusting the position of the nozzles; 18 – reflector.

The creation of the required milk pressure is achieved by the gas cylinder 1 and cylinder 11, which are connected by the air duct 3. The pressure is controlled by the pressure gauge 5. The discharge valve 2 is used to fill the cylinder with gas from the cylinder. The milk is poured into the cylinder using the funnel 7, the hose 8, and the filling valve 6. The outlet valve 4 is necessary for the release of gas from the cylinder when it is filled with milk. The piston 12 prevents the diffusion of gas into the milk and, thus, a change in its properties.

The cylinder and nozzles are connected by hydraulic hoses 14. The division of the main milk flow from the cylinder into two equal flows is carried out in the tee 9. The main valve 13 is used to supply milk under the required pressure to the nozzles.

When performing the tests, the required volume of milk was poured into the cylinder through the funnel, with filling and outlet valves open. The main valve was kept closed. To create the necessary pressure in the cylinder, a GOST 8050-85 carbon dioxide cylinder was used. After opening the discharge valve (outlet and filling valves closed), carbon dioxide was supplied to the cylinder and the pressure in it increased to the required value. The device uses a manometer with a measurement limit of 100 kg/cm^2, accuracy class 0.75 according to GOST 2405-85. When the main valve was opened, the milk under the required pressure was sent to the nozzles, in which jets were formed. After homogenization, the milk was gravitationally discharged through a hole in the lower part of the chamber.

The main factors of the experimental studies were the excess pressure of the milk supply to the nozzles Δp and the diameter of the nozzle cone d_n. The distance between the nozzles *a* was taken to be equal to half the diameter of the nozzle cone **(Samoichuk, 2008)**. The temperature of milk during homogenization was assumed to be 60 – 70 °C **(Walstra, Wouers and Geurts, 2006)**. For counter-jet homogenization, the excess pressure is related to the modified Weber criterion We^{o-s} by the ratio:

$$We^{o-s} = \frac{6\rho_p \cdot \varphi^2 \cdot \Delta p}{10^6 \sigma_{f-p} \cdot \rho_m}, \quad (1)$$

Where: ρ_p, ρ_m – density of milk plasma and milk, kg.m^{-3}; φ – hydraulic coefficient of jet speed; σ_{f-p} – surface tension between milk fat and plasma, N.m^{-1}; Δp – excess milk supply pressure, Pa.

For the experimental studies, whole milk was used **(DSTU 8553, 2015)**, with a density of 1027 – 1023 kg.m^{-3}, and fat content of 2.5 – 4.4%.

Statistical analysis

The degree of homogenization of milk was determined by the formula **(Loncin and Merson, 1979; Nuzhin and Gladushnyak, 2007)**:

$$Hm = \frac{d_0}{d_k}, \quad (2)$$

Where:

d_0, d_k – average diameter of fat globules before and after homogenization, μm.

Average diameters of fat globules and other dispersive indices of the milk emulsion were determined by computer analysis of the micrographs of milk samples obtained with an optical microscope and a Mustek Wcam 300 digital camera (resolution 640×480) **(Samoichuk et al., 2020)**. Each experiment was repeated three times. From each experiment, three samples were selected and two dilutions were prepared from each sample. Six characteristic microscope field of view photos were selected from each dilution. Thus, 36 microscope fields of view were analysed to determine the statistical characteristics of milk.

The geometric characteristics of fat globules were analysed based on digital image analysis of the micrographs obtained.

The number of fat globules in the microscope field of view and their diameter were determined in the process of calculations. The average diameter of fat globules was determined by the statistical method of power average (arithmetic mean).

The qualitative uniformity of the measured data set was estimated by the coefficient of variation V. For dispersed indicators of milk fat globules, the coefficient of variation is sufficient for V >0.6 **(Haponiuk, Zander and Probola, 2015; Di Marzo, Cree and Barbano, 2016)**. When this condition is satisfied, the values of V are not given separately.

For this purpose, a software module has been developed that is implemented in Microsoft Visual Studio 2010 based on C# using the OpenCV Sharp library set 4.2.0. The exported numerical data and calculation of the sample statistics were performed in Microsoft Office Excel 2010.

A McBrain VA 318 electric wattmeter (Volga Region Power Equipment Plant, Russia) was used to record power.

RESULTS AND DISCUSSION

The rationale for the design of the reflector

For efficient operation of the annular reflector, the liquid flows after their collision with the annular reflector mustn't intersect with the main jets of milk (coming out of the nozzles) **(Fialkova, 2006; Innings and Trägårdh, 2005)**. Otherwise, there will be a violation of the continuity of the main jets and a decrease in the degree of dispersion of milk emulsions. To meet these requirements, the annular reflector in the radial cross-section must have the shape of an equilateral triangle, the vertex of which faces inwards of the annular reflector. With this design, the jets reflected from the surfaces of the reflector are removed outside the main jets of milk, which is necessary to prevent their crossing (Figure 2).

To prevent the intersection of the main jets of milk with those reflected from the reflector, it is necessary to calculate the angle β (Figure 3).

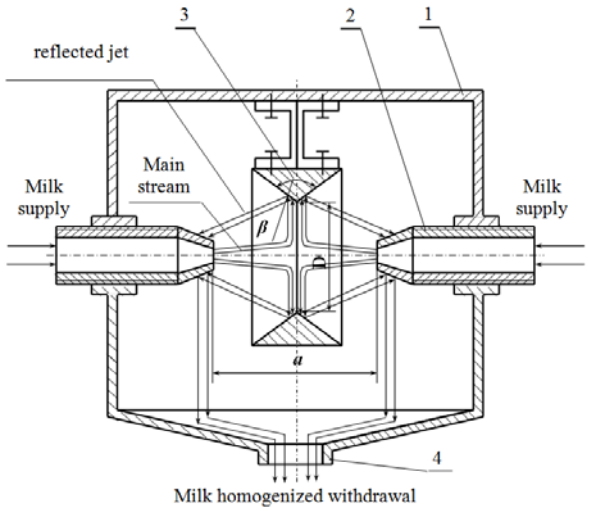


Figure 2 Scheme of the location of the annular reflector and direction of milk flows in the process of homogenization. Note: 1 – camera body; 2 – nozzle; 3 – annular reflector; 4 – hole for draining homogenized milk; D – diameter of the reflector; β – the angle of the reflector; *a* – the distance between the nozzles.

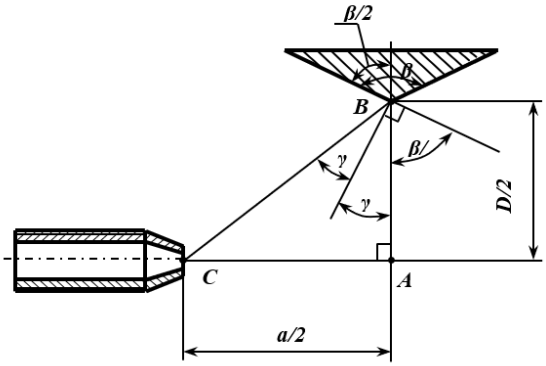


Figure 3 Calculation scheme for determining the angle β of the annular reflector.

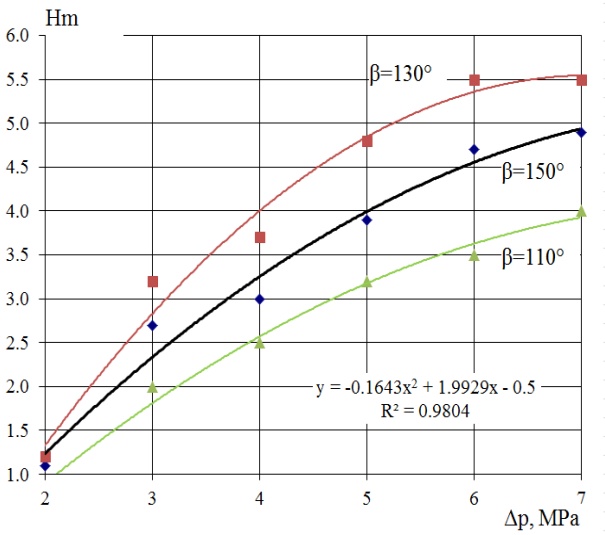


Figure 4 Dependence of the influence of excess pressure and angle of the reflector on the degree of homogenization at 70 °C.

$$\beta = 180^0 - \text{arctg}\frac{a}{D}. \quad (3)$$

The counter-jet homogenizer with a reflector works as follows. Whole milk is fed into the nozzles under the required pressure, which depends on the required degree of homogenization. After passing through the nozzle cones, the milk jets meet, while the fat fraction of milk is dispersed and mixed. The coaxial arrangement of the jets allows the fullest use of the kinetic energy of the fluxes for grinding the dispersed phase **(Huppertz, 2011; Samoichuk, 2008)**. After collision and homogenization, the product flow diverges in a fan shape perpendicular to the direction of the jets and hits the annular reflector. This results in the final grinding and partial mixing of the dispersed phase of the mixture **(Nuzhin and Gladushnyak, 2007)**. After contact with the annular reflector, the mixture is reflected from it and enters the housing of the homogenization device, where it is gravitationally removed from the machine. Moreover, due to the properly calculated angle of the annular reflector β, the product streams after contact with the annular reflector are reflected outside the jets coming out of the nozzles, so that there is no intersection of the flows of non-homogenized and homogenized products. Thus, in the counter-jet homogenizer with a reflector, dispersion of the fat phase of milk occurs in two stages: when the jets collide with each other and when the secondary jets collide with the reflector. This allows fuller use of the energy of the flow of milk.

Results of experimental studies

To determine the effect of excess pressure and the angle of the reflector on the degree of homogenization, an experiment was performed, the results of which are shown in Figure 4.

With increasing excess pressure, the degree of homogenization increases with parabolic dependence. At higher values of excess pressure, the growth of the response function (Hm) slows down. The prediction performed by the computer program Microsoft Office Excel 2010 **(Lawrence, Klimberg and Lawrence, 2009)** shows a maximum achievable degree of homogenization of 5.6 at an overpressure value of 7.4 – 7.5 MPa.

The optimal value of the angle of the reflector, calculated by the formula (3), is 130°. This achieves the highest degree of dispersion of the fat phase of milk – milk flow after reflection from the surfaces of the reflector is directed to the nozzle body and does not interfere with the free exit of the main jet from the nozzles. At an angle of the reflector β = 150°, the flow of milk strikes further on the body of the nozzles. In this case, due to the greater distance, the jet speed becomes smaller, so the efficiency of additional homogenization decreases. At an angle of the reflector β = 110°, the milk flow after the reflector intersects with the jets coming out of the nozzles, so the continuity of the milk flow is disturbed and its normal velocity component falls, so the decrease in the degree of homogenization is more significant.

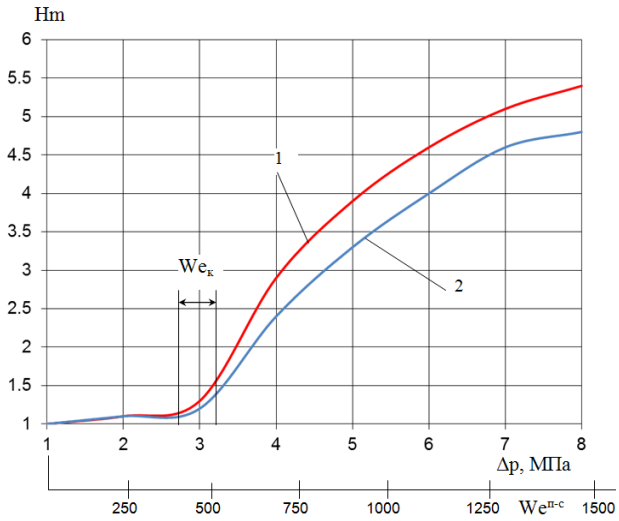


Figure 5 Comparison of the dependence of the degree of homogenization on excess pressure and the modified Weber criterion (at T = 70 °C, a = 0.56 mm, d_c = 1 mm): 1 – counter-jet homogenizer with a reflector; 2 – counter-jet homogenizer without a reflector.

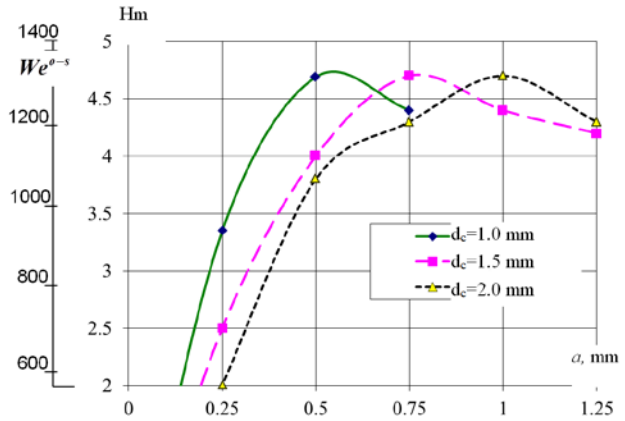


Figure 6 Dependence of the degree of homogenization and the modified Weber criterion on the distance between nozzle cones.

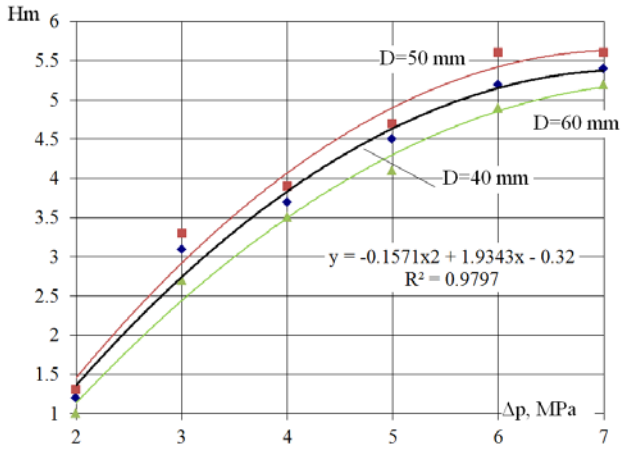


Figure 7 Results of experimental determination of the degree of homogenization depending on the pressure and diameter of the reflector.

The deviation of the values of the experimental curve of the modernized homogenizer in comparison to the homogenizer without a reflector is 15 – 20%. Indeed, the increase in the degree of homogenization with the reflector installed is due to more complete use of the kinetic energy of the milk flow in additional contact with the reflector and the nozzle housing.

Approximating the data of Figures 5 with a straight line, we obtain an expression that is identical in content to the known dispersion formula **(Loncin and Merson, 1979)**:

$$Hm = 0.9 \cdot 10^{-6} \Delta p \cdot \varphi^2 \qquad (4)$$

The coefficient of determination (R^2) in the range of 2.5 <Hm <6.0 is 95%, and at Δp = 5.0 – 6.0 MPa and a degree of homogenization of 4.5 – 5.2, the difference between theoretical and practical data is minimal. At Δp = 6.5 MPa, this difference reaches 6% and with a further increase in excess pressure, it is possible to predict its rapid increase. In the range Δp = 2 – 3 MPa, there is an intensive increase in the degree of grinding of the fat phase of milk. Here, the deviation of experimental data from the specified (approximated) dependence is maximal. At the value of Δp <2 MPa, homogenization practically does not occur.

The critical value of the Weber criterion (the beginning of the grinding of the fat phase) corresponds to the range of excess pressure of 1.8 – 2.2 MPa, at which We = 500 – 600.

Therefore, the optimal parameters of counter-jet homogenization for d_c = 1 mm are: a = 0.5 mm and T = 60 – 65 °C. The value of excess pressure depends on the required degree of homogenization and is Δp = 6.5 MPa at Hm = 5.0.

The results of experimental determination of the degree of homogenization and Weber criterion, with nozzle cone diameters of 1.0, 1.5, and 2.0 mm, depending on the distance between the nozzles cones, are shown in Figure 6. The diameter of the cone does not affect the maximum degree of homogenization.

It should be noted that at $a < d_c / 2$ the degree of homogenization is higher by 15 – 40% than that theoretically calculated **(Samoichuk, 2008)**, and the velocity of the jets at $a < d_c / 2$ corresponds to the calculated data. This can be explained by a more sudden change in the velocity of the fat globules after the collision of jets (which leads to an increase in the velocity difference between the fat globules and the surrounding plasma), due to the strict limitation of boundaries of the jet flow that is diverted by the edges of the nozzles **(Samoichuk and Kovalyov, 2013)**. Therefore, the optimal location of the nozzles is at a distance equal to half the diameter of the nozzle cone **(Samoichuk, 2008)**.

To determine the diameter of the annular reflector of the counter-jet homogenizer, the experimental study was conducted with reflector diameters D = 40, 50, and 60 mm. The results are shown in Figure 7.

It is optimal to use a reflector with a diameter of 50 mm as we obtain the maximum degree of fat dispersion. At D = 40 mm, the degree of homogenization decreases by 0.2 – 0.3, that is by 5%. This can be explained as follows. When using a reflector with a smaller diameter to comply

with the formula (3), the angle β decreases and the impact on the reflector becomes more sliding, which reduces the degree of homogenization. At D = 40 mm, the degree of homogenization decreases by 0.5 – 0.6, i.e. by 10%. This is due to the increase in loss of flow velocity before the collision with the reflector.

Changes in the fractional composition of fat globules after counter-jet homogenization **(Samoichuk, 2008)** (at T = 65 °C, Δp = 3.5 MPa) and comparing them with homogenization with a reflector (at a pressure of 4 MPa and T = 65 °C) and whole milk **(Dhankhar, 2014; Nuzhin and Gladushnyak, 2007; Oreshina, 2010; Haponiuk, Zander and Probola, 2015)** are graphically represented in Figure 8, and micrographs of fat globules are shown in Figure 9.

Milk before homogenization is characterized by the following parameters: average diameter of fat globules d_k = 2.49 mm, dispersion σ = 1.66, coefficient of variation

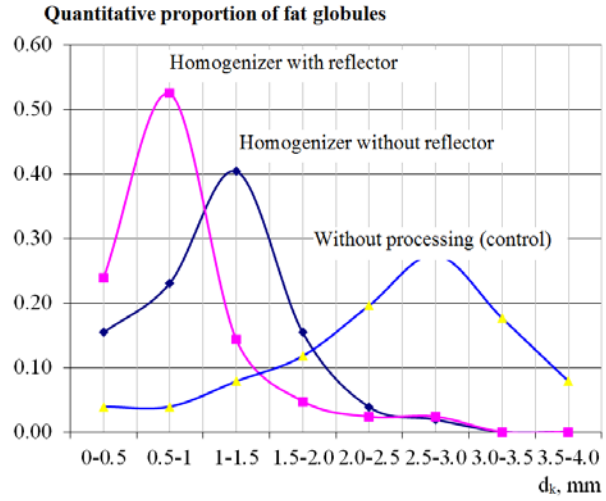
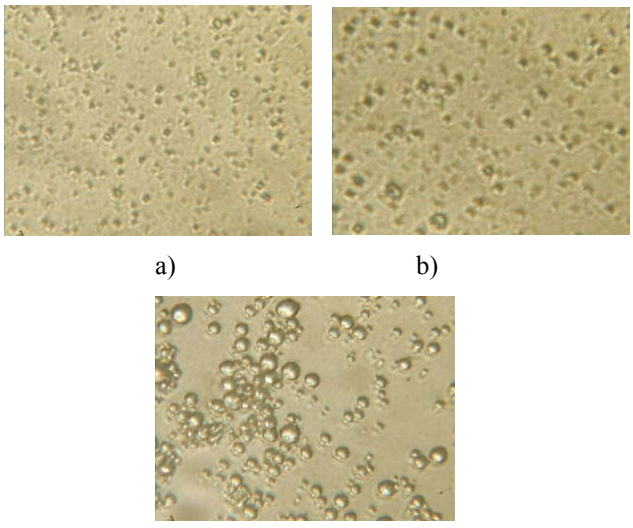


Figure 8 Differential distribution of fat globules of milk.



a) b)

c)

Figure 9 Photomicrographs of milk samples (400× magnification). Note: a) after counter-jet homogenization with a reflector at Δp = 4.0 MPa; b) after counter-jet homogenization at Δp = 4.0 MPa; c) raw milk.

(the share of scattering of the trait relative to the average) V = 67% **(Di Marzo, Cree and Barbano, 2016; Floury, Desrumaux and Lardieres, 2000; Hussain et al., 2017)**. Respectively for milk after counter-jet and countercurrent-jet homogenization with a reflector: d_k = 0.99 mm and 0.83 mm, σ = 0.51 and 0.47, V = 51% and 56%.

The value of the coefficients of variation indicates the reliability of the data sample.

The average diameter of the fat globules for the counter-jet homogenization treatment with the reflector decreased by 19% (from 0.99 to 0.83 μm) compared to that with the non-upgraded homogenizer. The dispersion value also decreased, which indicates the advantage of counter-jet homogenization with a reflector.

CONCLUSION

The design of the annular reflector has been developed to ensure the condition of free flow after collision with the surface of the reflector. The reliability of the theoretically obtained dependences for determining the angle of the reflector has been confirmed. It is proved that it is optimal to use a reflector diameter of about 50 mm.

When using a counter-jet homogenizer, it is possible to achieve a degree of homogenization of 5.6 at an excess pressure of 7.4 – 7.5 MPa. The degree of homogenization when installing an annular reflector increases by 15 – 20%. Moreover, such an increase in quality is provided without increasing energy consumption.

A comparison of the data on the distribution of milk fat globules by size after counter-jet homogenization and homogenization with a reflector suggests that the average diameter of fat globules for the experimental method is 19% smaller. The width of the particle size distribution is also smaller, which indicates better homogenization with the reflector installed.

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BRYNDZA CHEESE OF SLOVAK ORIGIN AS POTENTIAL RESOURCES OF PROBIOTIC BACTERIA

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ABSTRACT

Bryndza cheese includes several predominant lactic acid bacteria. The aim of our study was the antagonistic effect of lactic acid bacteria supernatant isolated from ewes´ cheese bryndza against ten Gram-positive and Gram-negative bacteria. Isolated strains of bacteria were obtained from bryndza which were produced in five different regions of Slovakia. Isolated strains of lactic acid bacteria were identified with mass spectrometry MALDI-TOF MS Biotyper. A total of one hundred and thirty lactic bacteria include *Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Lactobacillus brevis, Lactobacillus harbinensis, Lactobacillus johnsonii, Lactobacillus plantarum, Lactobacillus paracasei* ssp. *paracasei, Lactobacillus paraplantarum, Lactobacillus suebicus, Lactococcus lactis* ssp. *lactis, Lactococcus lactis,* and *Pediococcus acidilactici* were tested in this study against Gram-negative bacteria: *Escherichia coli* CCM 3988, *Klebsiella pneumoniae* CCM 2318, *Salmonella enterica* subsp. *enterica* CCM 3807, *Shigella sonnei* CCM 1373, *Yersinia enterocolitica* CCM 5671 and Gram-positive bacteria: *Bacillus thuringiensis* CCM 19, *Enterococcus faecalis* CCM 4224, *Listeria monocytogenes* CCM 4699, *Staphylococcus aureus* subsp. *aureus* CCM 2461, *Streptococcus pneumonia* CCM 4501 with agar diffusion method. Lactic acid bacteria showed activity 92% against *Yersinia enterocolitica*, 91% against *Klebsiella pneumoniae*, 88% against *Escherichia coli*, 84% against *Listeria monocytogenes*. The most effective bacteria against Gram-positive and Gram-negative bacteria tested was *Lactobacillus paracasei* ssp. *paracasei*.

Keywords: bryndza; Gram-positive and Gram-negative bacteria; lactic acid bacteria; probiotic effect; mass spectrometry

INTRODUCTION

Bryndza is a traditional Slovak natural white spreadable ripened cheese with Protected geographical indication status **(Commission Regulation (EC) No. 676/2008)**. Bryndza is made from ewes' or a mixture of ewes' and cows' milk and is a rich source of protein, vitamins, and minerals **(Toth et al., 2016)**. Bryndza is a dairy product that naturally contains a broad spectrum of microorganisms that has crucial importance on cheese properties, flavor, and aroma. Microorganisms are present during the whole process of cheese production, they are important in coagulation and ripening **(Andrade et al., 2008; Tilocca et al., 2020)**. Also, beneficial strains help to inhibit the growth of the pathogens and reduce the spoilage of the dairy products **(Arqués et al., 2015)**.

In previous research Gram-positive, Gram-negative bacteria, and yeasts were found and identified in bryndza **(Kačániová et al., 2019)**. The dominant group of bacteria in bryndza was lactic acid bacteria (LAB), mainly *Lactobacillus* species **(Kačániová et al., 2020)**. *Lactococcus, Pediococcus, Enterococcus, Streptococcus* were abundant in bryndza from different Slovak regions also **(Berta et al., 2009; Šaková et al., 2015; Sádecká et al., 2019)**. The probiotic properties of bacteria isolated from bryndza were observed in *Lactobacillus plantarum*, *Enterococcus faecium,* and *Enterococcus faecalis* strains. Researches claimed that these potentially probiotic strains can inhibit the growth of pathogenic bacteria, and some of them can survive in the acidic gastrointestinal environment, which is necessary for reaching the intestine of the host **(Belicová et al., 2011; Belicová et al., 2013)**.

Probiotic bacteria as an important part of intestinal microbiota helps regulate the immune responses, relieve the gastrointestinal tract dysfunction, alleviate the allergies, or lower cholesterol levels **(Dicks and Botes, 2010**; **Plaza-Diaz et al., 2019)**. Moreover, the anti-carcinogenic properties of probiotic bacteria have been described **(Zhong et al., 2014)**.

Our study aimed to evaluate lactic acid bacteria isolated from ewes´ bryndza and select the most active probiotic bacterial strain against pathogens and opportunistic pathogens.

Scientific hypothesis

Bryndza isolates possess probiotic activity. LAB can inhibit antagonistic activity against pathogens.

MATERIAL AND METHODOLOGY

Isolation of lactic acid bacteria

A total of 130 lactic acid bacteria were isolated from Slovak ewes´ bryndza. The bryndza samples were obtained from five producers in Slovakia. Before identification, the lactic acid bacteria colonies were subcultured on 90% of Trypton Soya agar and 10% of Main Rogosa (MRS) agar (Oxoid) at 30 °C for 18 – 24 h microaerobically. One colony of each bacterial isolate was selected for screening. Subsequently, an analysis of the bacteria identification was performed using the MALDI-TOF MS Biotyper.

Bacterial strains for testing

The bacterial strains of Gram-negative bacteria: *Escherichia coli* CCM 3988, *Klebsiella pneumoniae* CCM 2318, *Salmonella enterica* subsp. *enterica* CCM 3807, *Pseudomonas aeruginosa* CCM 1959, *Yersinia enterocolitica* CCM 5671 and five of Gram-positive bacteria: *Bacillus thuringiensis* CCM 19, *Micrococcus luteus* CCM 732*, Listeria monocytogenes* CCM 4699, *Staphylococcus aureus* subsp. *aureus* CCM 2461, *Streptococcus pneumoniae* CCM 4501 were obtained from the Czech collection of microorganisms (Brno).

Antibacterial activity of LAB isolate

The culture of lactic acid bacteria after 24 h of incubation in MRS broth (Oxoid, Basingstoke, UK) was centrifuged at 5500 g for 10 min at 4 °C and 2 mL of the supernatant was used for detection of antibacterial activity.

The well diffusion assay was used. Bacteria were spread on Petri dishes with MRS agar. LAB isolates were added into 6 mm diameter wells were created into the agar. The amounts of LAB and indicator bacteria were the same (100 µl, 10^8 CFU/mL) prepared from the broth culture of bacteria according to the 0.5 McFarland standard. After 48 h incubation at 37 °C in an aerophilically chamber, the inhibition zone diameter was measured for detection of the antagonistic effect of the LAB isolate against pathogenic bacteria.

Statistical analyses

The mean and standard deviation of inhibition zones was calculated for the detection of antagonistic effect against tested Gram-positive and Gram-negative bacteria.

RESULTS AND DISCUSSION

LAB antimicrobial products, such as bacteriocins, are very important in bioconservation of various foods. The diverse use of LAB bacteriocins, individually or as biopreservative combinations, may help to improve food safety, especially of traditional products **(Jamuna and Jeevaratnam, 2004; Mojgani and Amimia, 2007)**.

In our study 130, lactic acid bacteria isolated from ewes´ cheese bryndza (Table 1) were tested for antimicrobial activity and antagonistic effect against pathogenic gram-positive and Gram-negative bacteria.

Table 1 Number of LAB isolates.

Species of LAB	Number of isolates
Enterococcus faecalis	10
Enterococcus faecium	10
Enterococcus hirae	10
Lactobacillus brevis	10
Lactobacillus harbinensis	10
Lactobacillus johnsonii	10
Lactobacillus plantarum	10
Lactobacillus paracasei* ssp. *paracasei	10
Lactobacillus paraplantarum	10
Lactobacillus suebicus	10
Lactococcus lactis* ssp. *lactis	10
Lactococcus lactis	10
Pediococcus acidilactici	10
Total	130

In agar test, lactic acid bacteria isolated from ewes´ cheese bryndza demonstrated different antimicrobial activity with the inhibition the zone ranged from <1 to >5 mm. Altogether, 92% of LAB showed activity against *Yersinia enterocolitica*, 91% against *Klebsiella pneumoniae*, 88% against *Escherichia coli*, 84% against *Listeria monocytogenes*. Antimicrobial activity lower than 84% was observed against *Salmonella enterica* subsp. *enterica, Pseudomonas aeruginosa, Bacillus thuringiensis, Micrococcus luteus, Staphylococcus aureus* subsp. *aureus,* and *Streptococcus pneumoniae* (Table 2). **Belicová et al. (2013)** tested 125 acid resistant presumptive lactobacilli isolated from bryndza against *Listeria monocytogenes* CCM 4699, *Staphylococcus lentus* CCM 3472, *Acinetobacter calcoaceticus* CCM 4503, *Sphingomonas paucimobilis* CCM 3293, and *Salmonella enterica* subsp. *enterica*, serovar Typhimurium.

LAB produces metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetoin, carbon dioxide, and bacteriocins that were classified as antimicrobial agents. Moreover, the production of organic acids resulted in low pH, which also inhibits the activity of pathogenic microorganisms **(Ponce et al., 2008; Šušković et al., 2010)**. Antibacterial activity of organic acids and bacteriocins was confirmed against various pathogenic Gram-positive and Gram-negative microorganisms **(Maragkoudakis et al., 2009)**.

A total of 20 LAB isolates showed strong inhibition zones (more than 5 mm) against *P. aeruginosa* and *M. luteus*. Nineteen of LAB isolates showed strong antimicrobial activity against *S. aureus*. Cell suspension of *Lactobacillus plantarum* inhibited *L. monocytogenes* growth **(Ennahar et al., 1998)**. *Lactococcus lactis* reduced levels of *L. monocytogenes* in Cheddar cheese **(Buyong et al., 1998)**. Lactococci developed by **Reviriego et al. (2005)** and **Reviriego et al. (2007)** reduced the number of *L. innocua, L. monocytogenes, S. aureus*, and *E. coli* in cheese **(Rodríguez et al., 2005)**. The intermediate inhibitory effect against Gram-negative and Gram-positive bacteria (1 – 5 mm) is shown in Figure 1. The most effective strains were *Lactobacillus paracasei* ssp. *paracasei* against all tested pathogens. This bacteria exhibited inhibitory activity against all 10 bacteria tested with 2 to 8 isolated were active against particular bacteria.

Table 2 Number of lactic acid bacteria isolates with antimicrobial effect against pathogenic bacteria in mm.

Inhibition zone	EC	KP	SEE	PA	YE	BT	ML	LM	SAA	SP
<1	5	4	10	15	5	15	20	6	11	15
1 – 5	115	118	105	95	120	105	90	110	100	101
>5	10	8	15	20	5	10	20	14	19	14

Note: EC – *Escherichia coli*; KP – *Klebsiella pneumoniae*; SEE – *Salmonella enterica* subsp. *enterica*; PA – *Pseudomonas aeruginosa*; YE – *Yersinia enterocolitica*; BT – *Bacillus thuringiensis*; ML – *Micrococcus luteus*; LM – *Listeria monocytogenes*; SAA – *Staphylococcus aureus* subsp. *aureus*; SP – *Streptococcus pneumoniae*.

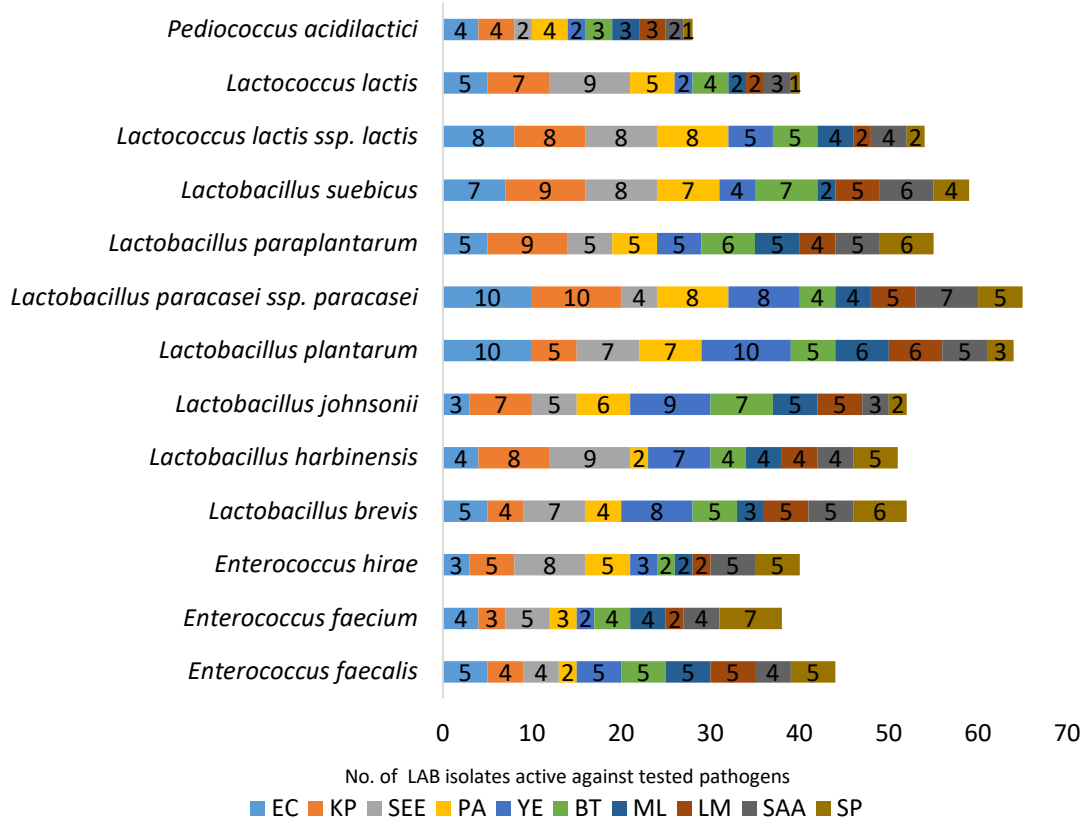

Figure 1 The inhibitory activity of the most active predictive lactic acid isolates against the pathogenic strains. Note: EC – *Escherichia coli*; KP – *Klebsiella pneumoniae*; SEE – *Salmonella enterica* subsp. *enterica*; PA – *Pseudomonas aeruginosa*; YE – *Yersinia enterocolitica*; BT – *Bacillus thuringiensis*; ML – *Micrococcus luteus*; LM – *Listeria monocytogenes*; SAA – *Staphylococcus aureus* subsp. *aureus*; SP – *Streptococcus pneumoniae.*

In total, inhibitory activity was expressed 64 times by 10 *L. paracasei* spp. *paracasei* isolates.

The most effective bacterial strain was *L. lactis* subsp. *lactis* against followed by *L. paraplantarum* against *S. enterica* subsp. *enterica*. The lowest antagonistic effect of lactic acid bacteria was found in *Enterococcus hirae* against *Bacillus thuringiensis* (Table 3).

Foodborne pathogens have become an important social topic and have received much attention from consumers and food safety regulatory agencies around the world because of frequent foodborne outbreaks. In previous studies, LAB showed a wide range of antimicrobial effects against many foodborne pathogens **(Soerjadi et al., 1981; Ennahar and Deschamps, 2000; Messens and De Vuyst, 2002; Dodd, 2012)**. Studies of interaction between LAB and
L. monocytogenes in various media and with various LAB strains have been performed, and in all cases LAB inhibited the growth of *L. monocytogenes* **(Tharrington and Sorrells, 1992; Zhang et al., 2011; Zhu et al., 2014)**.

However, **Kao and Frazier (1966)** obtained a mixed result when LAB was cocultured with *S. aureus*. Many researchers have found that LAB can inhibit *Salmonella* **(Keersmaecker et al., 2006; Zhang et al., 2016; Yang et al., 2017)** and that the bacteriostatic substances produced by LAB are thermally stable. The research focused on the inhibition of *E. coli* by LAB is more extensive compared to inhibition of the other bacteria. **Du et al. (2016)** found three strains of *Lactobacillus acidophilus* which could inhibit
E. coli ATCC 25922. Other investigations on *E. coli* O157:H7 proliferation control showed that LAB could effectively inhibit the growth of *E. coli* O157:H7 **(Brashears et al., 1998; Fooladi et al., 2014)**. *Klebsiella pneumonia, Bacillus cereus, Shigella flexneri* **(Sharma et al., 2017)**, *Staphylococcus epidermidis* **(Diepers et al., 2016)**, and *Candida albicans* **(Yu et al., 2006)** also can be inhibited by LAB.

Table 3 Antagonistic effect of lactic acid bacteria against Gram-negative and Gram-positive bacteria in mm (mean=zone of inhibition of 10 isolates ±*SD*).

LAB	EC	KP	SEE	PA	YE	BT	ML	LM	SAA	SP
Enterococcus faecalis	3.70 ±2.31	3.70 ±1.25	3.30 ±2.06	3.30 ±1.16	3.60 ±1.07	2.40 ±1.07	2.70 ±1.16	2.90 ±0.87	2.50 ±0.53	2.40 ±0.70
Enterococcus faecium	3.90 ±1.73	3.40 ±1.51	3.10 ±2.08	3.40 ±1.00	3.20 ±1.23	2.50 ±0.97	2.60 ±0.97	2.60 ±0.52	2.50 ±1.18	2.50 ±1.08
Enterococcus hirae	3.40 ±1.78	3.20 ±1.40	3.00 ±2.00	3.20 ±1.03	3.00 ±0.82	2.00 ±0.47	2.50 ±0.85	2.70 ±0.68	2.20 ±1.14	2.70 ±1.34
Lactobacillus brevis	4.10 ±1.79	3.80 ±2.10	3.70 ±2.41	3.80 ±2.10	4.40 ±0.97	2.60 ±1.07	2.70 ±0.67	2.90 ±0.74	2.60 ±1.35	3.00 ±1.70
Lactobacillus harbinensis	4.30 ±1.49	4.20 ±1.87	4.70 ±1.06	4.10 ±2.02	4.60 ±0.84	3.00 ±1.15	3.00 ±0.47	3.00 ±0.67	3.00 ±1.41	3.40 ±2.32
Lactobacillus johnsonii	4.60 ±1.27	4.40 ±1.58	4.20 ±2.30	4.40 ±2.07	4.70 ±0.67	2.70 ±1.25	3.10 ±0.32	3.20 ±0.42	3.50 ±1.51	3.30 ±2.54
Lactobacillus plantarum	4.70 ±1.06	5.10 ±1.73	4.50 ±2.01	4.70 ±2.06	4.80 ±0.42	2.90 ±1.10	3.30 ±0.67	3.10 ±0.57	3.60 ±1.35	3.50 ±2.42
Lactobacillus paracasei* ssp. *paracasei	4.90 ±0.88	5.30 ±1.57	5.10 ±2.08	4.90 ±1.85	4.90 ±0.31	3.00 ±1.05	3.60 ±1.07	3.40 ±0.70	3.80 ±1.69	3.50 ±2.84
Lactobacillus paraplantarum	5.00 ±0.67	5.40 ±1.58	5.60 ±1.84	4.80 ±1.81	4.80 ±0.42	3.20 ±1.03	3.70 ±1.06	3.60 ±0.84	3.90 ±1.66	3.70 ±2.87
Lactobacillus suebicus	4.80 ±0.63	5.30 ±1.57	5.00 ±1.76	4.50 ±1.18	4.90 ±0.57	3.30 ±0.95	3.50 ±0.71	3.50 ±0.71	4.00 ±1.56	3.80 ±2.78
Lactococcus lactis* ssp. *lactis	5.10 ±0.74	5.50 ±1.65	5.70 ±1.70	4.70 ±1.25	5.00 ±0.67	3.40 ±0.84	3.60 ±1.35	3.40 ±0.84	3.90 ±1.45	3.80 ±2.62
Lactococcus lactis	4.90 ±0.99	5.30 ±1.57	5.40 ±1.71	4.40 ±1.27	4.80 ±0.92	3.30 ±0.82	3.60 ±1.51	3.20 ±1.14	3.90 ±1.37	3.90 ±2.51
Pediococcus acidilactici	4.40 ±1.51	4.60 ±1.51	4.30 ±1.83	4.10 ±1.20	4.40 ±1.51	3.00 ±1.05	3.00 ±0.94	2.90 ±1.29	3.30 ±1.16	3.20 ±2.20

Note: EC – *Escherichia coli*; KP – *Klebsiella pneumoniae*; SEE – *Salmonella enterica* subsp. *enterica*; PA – *Pseudomonas aeruginosa*; YE – *Yersinia enterocolitica*; BT – *Bacillus thuringiensis*; ML – *Micrococcus luteus*; LM – *Listeria monocytogenes*; SAA – *Staphylococcus aureus* subsp. *aureus*; SP – *Streptococcus pneumoniae.*

CONCLUSION

In conclusion, the present study of lactic acid bacteria strains isolated from ewes´ cheese bryndza confirmed antagonistic effect against Gram-positive and Gram-negative bacteria.

In our study, the best results were found for *Lactococcus lactis* subsp. *lactis* against *Salmonella enterica* subsp. *enterica*. *In vitro* screening of LAB from Slovak bryndza ewes´ cheese have good potential for use as probiotic cultures.

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ANALYSIS OF THE POSSIBILITY OF FISH AND MEAT RAW MATERIALS COMBINATION IN PRODUCTS

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ABSTRACT

Aspects for the use of regional raw materials in ground food technology require further study of functional and technological properties to be able to predict them depending on the chemical composition of raw materials and processing methods. The aim of our research was to comparatively study the chemical composition, functional-technological, rheological properties of fish raw materials, and duck meat in terms of their possible compatibility in meat-containing products. The subject of our research was representatives of the regional aquaculture *Carassius gibelio* and *Hypophthalmichthys molitrix*, as well as the meat of Muscovy duck (*Cairina moschata*). It has been established that the nutritional value of freshwater aquaculture objects *Carassius gibelio* and *Hypophthalmichthys molitrix* is virtually identical in protein and fat content, making them interchangeable in terms of nutritional balance when developing the combined product. The ratio of protein and fat to water for duck meat is higher 3.54 – 4.88 times that of aquaculture, which can be used in the selection of components of the formulation of emulsified products, taking into account the nutrient balance. It has been confirmed that the addition of salt enhances water holding capacity, water binding capacity, and fat holding capacity. Water holding and water-binding capacities of minced fish are higher due to higher water levels, which, combined with the low-watering duck meat, can be predicted to create a forcemeat system with high functional-technological properties. The ability to emulsification and retain fat in the state of emulsion in minced duck meat has proved to be better, which when combined with fish minced meat can compensate for the ability to retain fat in the system of combined products. A combination of regional aquaculture with waterfowl meat will not only improve the functional and technological parameters of combined minced systems but also balance them by correcting the composition of proteins and fats.

Keywords: fish; meat; combination; analysis

INTRODUCTION

The problem of healthy nutrition is one of the most important issues facing society. Human health depends on the satisfaction of physiological needs for energy and nutrients **(Gibney et al., 2009**; **Waterlander et al., 2018)**. According to the WHO Regional Office for Europe, about 80% of all diseases are in one way or another related to nutrition, and for 41% of them, nutrition is a leading risk factor **(WHO, 2004)**. Violation of dietary recommendations leads to the development of pathological conditions of the body, especially with prolonged deviation from a balanced diet **(Hennig et al., 2018; Kimokoti and Millen**, **2016)**.

One of the reasons for the unfavorable conditions for human health is the lack of protein in the diet, which today is up to 30% for Ukrainians, especially for the protein of animal origin **(Prokopenko**, **2018)**. The main sources of animal protein are raw meat from slaughtering cattle and pigs. The volume of their production no longer meets the needs of the world population in protein and, therefore, in essential amino acids. Moreover, the methods used in agriculture for meat production have almost exhausted their potential **(FAO, 2018)**.

Aquaculture is of practical interest in filling nutrient deficiencies as promising components for creating new foods. The use of fish in conjunction with raw meat will significantly expand and diversify the range of high-quality protein products of high quality and create the possibility of designing products with a balanced nutritional composition. Furthermore, combining animal protein of different origins, in addition to the rational use of raw materials, will allow expanding the volume of biologically complete protein products, to ensure the economic efficiency of its production, by reducing the cost.

On the one hand, the range of meat products has been significantly expanded in recent years, in the formulation of which different ingredients of non-meat origin are used. On the other hand, aquaculture production has become an important source of food protein in many countries over the last decades **(Pauly and Zeller, 2017)**. This industry is developing rapidly, and its competitiveness is driven by the availability of raw materials to processing facilities, its rapid renewal, and low cost.

The nutritional and biological value of fish raw materials is the main indicator of its inclusion in the formulation of dietary foods. In the proteins of the muscle tissue of the hydrobionts, the high content of the compounds necessary for the human body, such as essential amino acids, polyunsaturated fatty acids, including the unique ones - docosahexaenoic and eicosapentaenoic **(Mohanty et al., 2016)**. Fish ranks first among animal protein products by methionine content **(Vidotti et al., 2003)**.

In addition to resource availability, an important criterion for the selection of aquaculture in food technology is its nutritional value, organoleptic, taste, and functional and technological characteristics. A sufficient number of works are devoted to the study of the chemical composition of freshwater aquaculture **(Abramova, 2005; Lebsjka and Gholembovsjka, 2014; Bozhko et al., 2018; Boghdanov, 2005)**.

In addition to the traditional indicators of the biological and nutritional value of fishery raw materials, **Tereschenko (2004)** suggested to supplement their characteristics with some critical coefficients and, first of all, with the coefficient of nutritional saturation (C_{ns}). According to the classification of **Tereschenko (2004)** fishery raw materials, depending on C_{ns} is divided into low saturated ($C_{ns} \leq 0.3$), medium saturated ($C_{ns} = 0.3 – 0.6$), and highly saturated ($C_{ns} > 0.6 – 1.5$).

As a result of studies of the biological efficiency, nutritional and biological value of fish muscle tissue, fish proteins have been found to have a high digestibility rate of 1.89 – 1.90, while for beef this figure is only 1.64 units **(Sidorenko, 2009)**.

However, there is a problem with aquaculture implementation, which is related to the peculiarities of the anatomical structure of the fish body. The presence of a large number of soft muscular bones, as well as the specific taste, necessitate the processing of fish for minced meat and combine it with other raw materials. At the same time, due to the wide variation of the components of the stuffing, it is possible to manufacture a wide range of products, including those with high biological value and certain physiological orientation **(Bozhko et al., 2018)**. Combining raw materials with different functional and technological properties (FTV) makes it possible to obtain products with a wide range of functional properties.

One of the pressing issues in the technology of combined food is the use of raw materials, which under the influence of technological processes forms a homogeneous system with directional-set properties. The production of high-quality products with a combined composition of raw materials requires information about the kinetics of the structure formation process, hydrodynamic properties of raw materials, which determine the degree of bound moisture, and, therefore, technological and consumer performance of products. Simultaneously selected components of the formulation should have sufficient technological properties, their maximum compatibility, or mutual compensation to ensure stable food emulsions.

At the same time, at each stage of the technological process, they take into account the characteristic functional properties of each ingredient and the influence of each of them on the formation of a stable emulsion and the qualitative characteristics of the finished products **(Kosoy et al., 2005)**. Thus, the prospects for the use of regional aquaculture in ground-based food technology require further study of functional and technological properties to be able to predict them, depending on the chemical composition of the raw material and the processing methods used.

Scientific hypothesis

We are expecting the confirmation of the high technological qualities of fish raw materials and the possibility of combining it with meat raw materials as part of the combined products. Therefore, the purpose of the research was to comparatively study the chemical composition, functional-technological, rheological properties of fish, and meat raw materials in terms of their possible compatibility in meat-containing products.

We are expecting that the chemical composition, high functional and rheological indicators, critical coefficients of *Carassius gibelio,* and Muscovy duck meat will improve the technological properties of combined meat systems, balance them by correcting the composition of proteins and fats.

MATERIAL AND METHODOLOGY

The subject of our research was representatives of the regional aquaculture *Carassius gibelio* and *Hypophthalmichthys molitrix*, as well as the meat of Muscovy duck.

Carassius gibelio weighing 250 – 350 g and a white carp (*Hypophthalmichthys molitrix*) weighing 1800 – 2000 g were purchased from the local supermarket in Sumy, Ukraine. The fish was stripped of the scales, the interior was removed, the meat was separated from the bones and ground into a meat grinder with a grid diameter of 2 – 3 mm. Samples of minced meat were divided into two groups, one of which was added 1.5% salt to the minced meat.

Muscovy duck (*Hypophthalmichthys molitrix*) weighing 2000 – 2500 g was purchased from the Sumy region local market from a poultry farm. Ducks were deboned and ground. For processing, the duck meat was minced through a 2 mm plate and then through a 3 mm plate of a meat grinder. Two types of forcemeat were prepared: 1 – without salt, 2 – with an addition of 1.5 % salt. The salt was added during mixing the minced meat. Samples were evaluated for different parameters.

Raw Protein Measurement

Protein measurements were performed using the Kjeldahl method **(ISO 937, 2007)**. 5 g of homogeneous fillet with 20 mL of concentrated sulfuric acid and 8 g of catalysts were placed in a special container and then heated at 350 °C for 30 min. After mineralization, the sample was quantitatively transferred to a solution of

NaOH at a concentration of 33%, sealed, and distilled off with the steam. The resulting steam distillate was transferred to a container containing several drops of the Tashiro indicator. The titration was performed with a solution of 0.01 N sulfuric acid.

Fat Measurement

Total fat was measured by the Soxhlet method **(ISO 1443, 2008)**. 4 g of the dried sample in a paper cartridge was placed in an extraction flask of a Soxhlet apparatus. Petroleum ether with a boiling point of 45 °C was used for the extraction. After multiple extractions, the weight of the test cartridge to constant weight was determined. The difference between the initial and final weight shows the percentage of fat.

pH measurement

The pH of the mincemeat was measured using a Partabell digital pH meter pcd650. Samples were prepared to measure pH based on the standard method **(Pasichnyi, 2013)**, and 10 g of minced meat in 100 mL of water were mixed.

Moisture analysis

Moisture was determined by the method of drying **(ISO 1442, 2008)**. 5 g of the sample was placed in a container, dried for 1 hour at 150 °C.

Cooking loss

Cooking losses were calculated by the difference of weight before and after cooking, and the moisture content was determined by drying the samples (4 g) at 150 °C.

Methods of measuring functional indicators

WBC (water binding capacity) of minced meat was determined by the pressing method **(Pasichnyi, 2013)**. WHC (water holding capacity) of minced meat was defined as the difference between the mass fraction of moisture in the minced meat and the amount of moisture released during the heat treatment. The following procedure was used to determine the emulsifying capacity (EC). The stability of the emulsion was determined by heating at 80 °C for 30 min. and cooling with water for 15 min followed by centrifugation and measuring the ratio of the emulsion layers **(Pasichnyi, 2013)**.

Determination of critical coefficients

Critical coefficients were determined by the method **(Kosoy et al., 2005)**. Coefficient of protein watering (Cw) was calculated as a ratio between moisture content and total protein; protein-water factor (PWF) as the ratio between total protein and moisture content; lipid-protein coefficient (LPC) - as the ratio between fat content and protein content; food saturation factor (FSF) as total protein, total fat and moisture content.

The coefficient of chemical composition (CCC) was calculated according to the formula (1):

$$CCC = [F/M] \times P \qquad (1)$$

Where: F – total fat, %; M – moisture content, %; P – total protein, %.

Definition of rheological indicators

Rheological indices of minced systems were determined using a rotational viscometer. RV-8m viscometer was used with a corrugated rotor (2 mm corrugation step) with an inner cylinder (Rc) of 0.605 cm, and an outer rotor radius of Rn – 1.9 cm, the length of the rotor was equal to 8 cm. on a scale using a stopwatch. The processing of the obtained results was performed according to the method **(Pasichnyi, 2013)**.

Statistical analysis

The data statistical analysis was produced by Microsoft excel and Statistica 15. All experiments were carried out in triplicate and the results reported are the results of those replicate determinations with standard deviations. The Student t-test was used for the statistical analysis of the obtained results. Data are presented as mean ±standard error of the mean (SEM). The smallest acceptable difference for probes from the one sample was pointed at 5%. Probes with more differences were not considered.

RESULTS AND DISCUSSION

Adherence to the theory of balanced nutrition has allowed substantiating a holistic system for determining the nutritional and biological value of products based on their chemical composition **(Pasichnyi, 2002)**. In turn, based on chemical composition data of food introduced the concept of different value categories. The term "nutritional value of the product" is used to determine the percentage of chemical composition conformity of the product under study to the formula of balanced nutrition, that is, the term reflects the completeness of the useful qualities of the product **(Bohrer, 2017; Sándor et al., 2011)**. On the other hand, the chemical composition of raw materials, namely the quantity and quality of proteins, the content of fat fraction and water, are responsible for the functional and technological properties of the food products created from it **(Kinsella, 1982; Sikorski, 2001)**.

In the development of combined products, the key is the selection of recipe composition, which is carried out on the basis of the chemical composition of raw materials and taking into account the compatibility of components from a technological point of view. Therefore, the first step in developing a new product is to analyze the nutritional value and chemical composition of the raw ingredients.

The results of the study of the chemical composition of two fish species: silver carp (*Carassius gibelio*) and silver carp white (*Hypophthalmichthys molitrix*) and Muscovy duck (*Cairina moschata*), presented in table 1.

The analysis of the table shows that the freshwater fish studied belong to the low-fat protein raw material. The crude protein content of the muscle of the silver carp was 18.60 – 18.78% depending on the season of the catch. Protein concentration in silver carp meat was slightly lower and amounted to 17.60 – 17.70%. High levels of protein in muscle in both marine and freshwater aquaculture have been identified by several researchers **(Tacon and Metian, 2013)**. Also, the results of studies **(Pilon et al., 2011; Rudkowska et al., 2010)** confirm the benefits of fish protein in human nutrition.

Protein and fat content in the muscle tissue of aquaculture and duck turned out to be almost identical.

Thus, the concentration of crude protein in duck muscle was 17.36 ±0.09%. Similar data were obtained by **(Mazanowski, Kisiel and Gornowicz, 2003)**. The crude fat of fish and duck muscles was almost the same, at 3.20 – 3.60%, while the total fat of the silver carp was 25.9% higher on average. The moisture content of all three study object objects varied between 75 – 79%, which is described by authors **(Ali, et al., 2007; Tilami and Sampels, 2018)**.

Moisture and protein content in muscle tissue determines the consistency, taste, and yield of the finished product. The moisture content also has a significant effect on the structure of the functional groups of the protein molecules, their stabilization and space configuration, and, thus, on the functional and technological properties of the meat system as a whole **(Huff-Lonergan and Lonergan, 2005)**. A certain prediction of the functional and technological properties of the muscle tissue of aquaculture objects can be made by the indicators of structural-critical coefficients, as C_w, PWF, and LPC. They complement the overall picture of rheological properties, such as effective viscosity. The chemical composition coefficient and the food saturation factor allow us to determine the technological suitability of the resulting mince and to determine possible variants of its combination or rational enrichment.

Table 2 shows the results of the coefficient calculation based on the study of the chemical composition of freshwater fish and Muscovy duck.

C_w of muscle tissue proteins of *Carassius gibelio* is 7.3% and 2.3% higher, depending on the season of catching than the silver carp. This indicates that in muscle tissue of *Carassius gibelio* one part of the protein accounts from 4.31 to 4.36 weight parts of moisture, most of which are insufficiently bound by hydrophilic protein components. Such muscle tissue is more elastic and gel-forming, as well as heat and mechanical resistance, which should be taken into account when selecting the processing method and modes. The moisture and protein ratio in the duck muscles is more balanced and is 3.73.

LPC of the muscle tissue of both the silver carp and the white silver carp had no significant differences. At the same time, some dependence of the values of this indicator on the catching season is traced. Both aquaculture species had LPC above in the autumn catching period, which also indicates a more tender and dense consistency of muscle tissue. Also, the value of LPC can predict the food saturation of raw materials. For duck meat, this figure is more than 3.54 – 4.88 times, which can be used in the selection of components of the formulation of emulsified products, taking into account the balance of nutrients.

The protein-water factor of the muscle tissue of the silver and white silver carp is in the range of 23.17 to 24.4, which indicates the ability to produce products with a gentle and juicy texture. On the other hand, the value of this figure for duck meat is 26.79, which indicates a denser texture. Therefore, the investigated raw material by the size of the PWF is close to the raw material with high molding properties, for which the value of the specified coefficient is normalized in the range from 26 to 30 units.

The calculation of the coefficients of the chemical composition and food saturation showed that the raw material is medium saturated, for which the value of this parameter is determined within 0.3 – 0.6 **(Tischenko, Bozhko and Pasichnyi, 2017)**.

Minced meat is a complex heterogeneous system. Its functional and technological properties depend on the ratio of tissues and their content of specific proteins, fats, moisture, and added components.

Sodium chloride content is one of the main components of the ground beef systems that affect the functional properties of proteins. Its contents and the way of incorporating it into the structure may have different effects on the water-binding capacity of the ground meat systems and, accordingly, on the organoleptic characteristics and yield of the finished products **(Hermansson and Akesson, 1975)**.

The ability of the muscle tissue of fish to bind and hold moisture depends on many factors, including storage temperature, pH, degree of grinding, and more. The size of

Table 1 The chemical composition of the muscle tissue of aquaculture and waterfowl.

Sample	Mousture, %	Protein, %	Fat, %	Ach, %
		Hypophthalmichthys molitrix		
Autumn catch	75.00 ±0.71	18.78 ±0.03	5.03 ±0.01	1.19 ±0.01
Spring and summer catch	76.10 ±0.46	18.60 ±0.04	4.21 ±0.03	1.09 ±0.01
		Carassius gibelio		
Autumn catch	78.80 ±0.36	17.60 ±0.07	3.60 ±0.17	1.20 ±0.03
Spring and summer catch	77.20 ±0.70	17.70 ±0.01	3.20 ±0.00	1.90 ±0.01
		Cairina moschata		
-	76.34 ±0.86	17.36 ±0.09	3.42 ±0.98	0.99 ±0.17

Table 2 Critical coefficients of muscle tissue of aquaculture and waterfowl.

Sample	Season of catching	C_w	PWF	LPC	CCC	FSF
Carassius gibelio	Autumn	4.36	23.17	0.207	0.96	0.28
	Spring and summer	4.31	23.10	0.285	0.77	0.27
Hypophthalmichthys molitrix	Autumn	3.99	22.40	0.267	1,25	0.32
	Spring and summer	4.09	22.30	0.226	1.09	0.30
Cairina moschata	-	3.73	26.79	1.01	4.66	0.53

the WHC muscle tissue is determined by the content of immobilized moisture in it.

Maintaining of WHC of fish meat in the process of processing at the level of fresh fish indicators is of great practical importance, as it allows increasing the yield and improving the quality of finished products.

With the increase of WHC, the stickiness and elasticity of the forcemeat increases, the shear stress decreases, and the functional properties improve **(Borresen and Alsted, 1983)**.

Salt gives the ground meat some flavor and improves rheological characteristics **(Smith, 2001)**. Thus, the introduction of up to 3% NaCl mincemeat mass increases the solubility of myosin-type proteins **(Bandman, 1999; Chen et al., 2017)**.

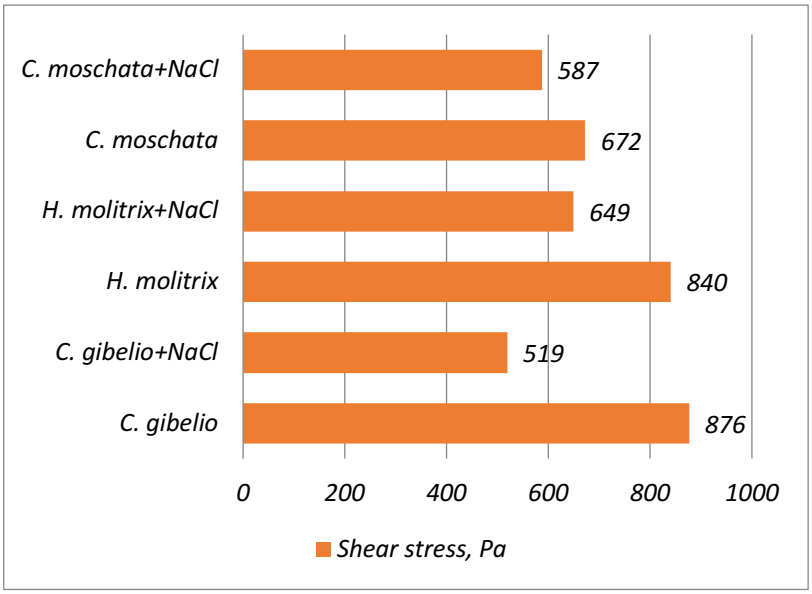

Figure 1 Shear stress of experimental samples forcemeat.

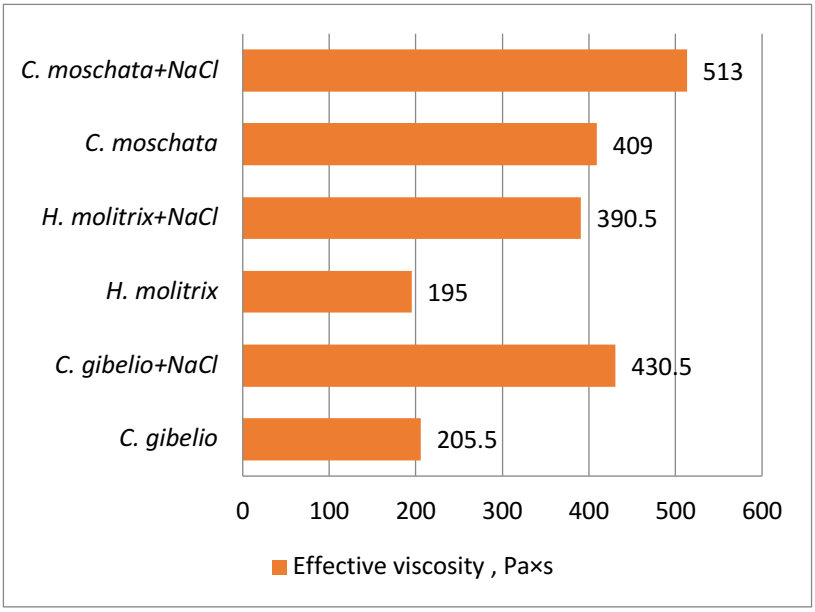

Figure 2 Effective viscosity of experimental forcemeat samples.

Table 3 Functional and technological properties of the muscle tissue of the studied aquaculture and waterfowl.

Parameter	*Carassius gibelio*		*Hypophthalmichthys molitrix*		*Cairina moschata*	
	forcemeat	forcemeat+ NaCl	forcemeat	forcemeat+ NaCl	forcemeat	forcemeat+ NaCl
Mousture, %	76.70 ±0.56	76.20 ±0.37	77.40 ±0.30	77.80 ±0.60	65.02 ±1.03	64.70 ±0.67
WHC, %	63.40 ±0.71	79.30 ±0.50	60.70 ±0.27	73.60 ±0.81	61.54 ±0.33	64.81 ±0.67
WBC_a, %	81.70 ±0.23	91.40 ±0.40	76.80 ±0.31	83.40 ±0.27	76.88 ±0.42	83.27 ±0.36
WBC_m, %	80.40 ±0.36	85.10 ±0.23	79.40 ±0.12	79.70 ±0.51	45.56 ±0.36	49.87 ±0.93
FHC, %	28.70 ±0.70	31.40 ±0.04	29.60 ±0.09	30.10 ±0.16	47.71 ±0.66	55.38 ±0.91
pH	6.47 ±0.03	6.51 ±0.01	6.31 ±0.07	6.37 ±0.03	6.21 ±0.03	6.38 ±0.01
Plasticity, $cm^2.g^{-1}$	9.40 ±0.93	12.70 ±0.77	8.70 ±0.20	10.51 ±0.30	15.54 ±0.03	14.87 ±0.07

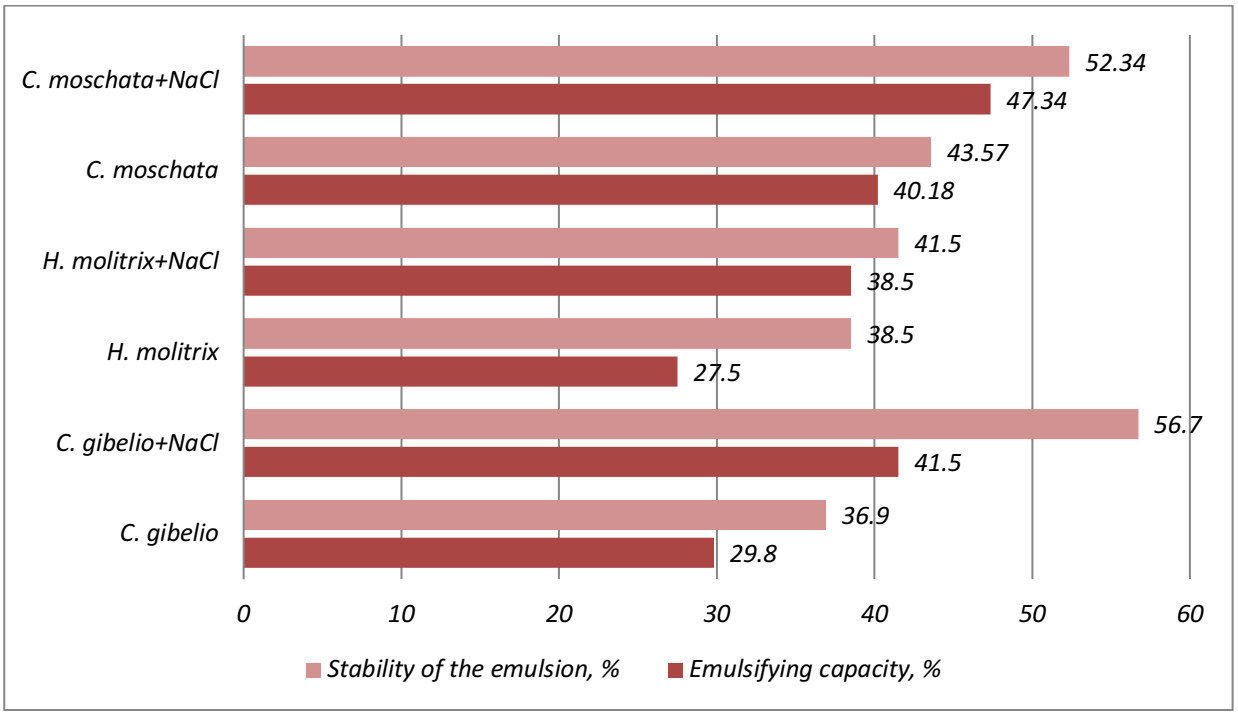

Figure 3 Emulsifying properties of experimental forcemeat samples.

At the same time, it is not recommended to introduce less than 1% NaCl into minced meat, since in this case, it acts as an oxidant **(Tischenko, Bozhko and Pasichnyi, 2016; Mariutti and Bragagnolo, 2017)**.

Table 3 summarizes the results of the functional properties study of minced meat of freshwater aquaculture and waterfowl.

The analysis of the table shows that the introduction into the structure of minced meat of 1.5% of salt has influenced the indicators of WHC and WBC.

The slightly higher indices of moisture-binding and moisture-holding capacity of minced meat in the presence of NaCl can be explained by the increased hydration of muscle tissue. This occurs by a mechanism where functional groups of proteins with electrostatic properties attract water dipoles and thereby increase hydration and WHC. **Ruusunen and Puolanne (2005)** explained in the work that chlorine ions, joining positively charged groups of proteins, support them in a state of swelling.

Good molding properties have forcemeat systems with WHC above 53%. All experimental samples of minced meat had an index of WHC by 10.7 – 13.9% higher than the minimum value, which is consistent by **Petrova and Bogdanov (2019)**.

Shear stress and effective viscosity of aquaculture and duck meat are shown in Figure 1 and Figure 2.

Plastic fluidity of forcemeat with the addition of salt begins at the size of the shear stress 519 Pa of muscle tissue of the crucian, which is 39.8% lower than in forcemeat without salt. It should be noted that there is a general tendency to improve the elastic-mechanical properties of minced meat with the addition of sodium chloride. The lowest viscosity of 195.0 and 205.5 Pa.s are minced without the addition of NaCl, indicating a profound disturbance of their structure and smear consistency.

When mixed with forcemeat 1.5% sodium chloride, there is a significant increase in their viscosity. Thus, the effective viscosity of *Carassius gibelio* muscle tissue increased by 42.8% and amounted to 430.5 Pa·s, and forcemeat of white carp – by 92.2% and became 390.5 Pa·s. The effective viscosity of duck meat was higher than fish and was 409 – 513 Pa·s, increasing with the addition of salt. So, it makes sense to combine fish forcemeat with waterfowl to increase effective viscosity, especially in the presence of NaCl. Analysis of rheological processes in minced meat will allow the use of the results obtained to evaluate and plan the technological processes in the production of combined food.

Results of the physical properties study of hydrobiota minced meat compared to waterfowl meat is shown in Figure 3.

The higher emulsifying capacity of the muscle tissue of *Carassius gibelio* is due to the values of the coefficients C_w and PWF, as well as the content of bound moisture. EC and SE of forcemeat are higher when NaCl is added. The values of these indicators are consistent with the indicators of WHC and WBC of minced fish and are caused by the increased content of water-soluble and salt-soluble fractions of proteins of the muscle tissue of hydrobionts **(Rembeza and Rechina, 1990)**. For this reason, only mobile and flexible macromolecules of proteins can form adsorption layers at the interface of two phases and to form a helical structure of the gel in a continuous phase **(Xiong and Brekke, 1989)**. According to **(Yakubchak, 2006)** the emulsifying capacity of minced poultry meat is 75%, and the emulsion stability is about 70%, which when combined with this raw material with multicomponent functional mixtures based on animal proteins **(Strashynskiy et al., 2016; Yancheva, Dromenko and Grinchenko, 2017)** will allow to effectively develop meat products with a combined composition raw materials.

CONCLUSION

It has been established that the nutritional value of freshwater aquaculture objects, namely *Carassius gibelio* and Muscovy duck, is identical in protein and fat content, making them interchangeable in nutritional balance when developing the combined product.

The ratio of protein, fat, and water for duck meat is higher 3.54 – 4.88 times then for aquaculture, which can be used in the selection of components for the formulation of emulsified products, taking into account the nutrient balance. The protein-water factor of muscle tissue of *Carassius gibelio* and white carp is in the range from 23.17 to 24.4, which indicates the possibility to produce products with a gentle and juicy consistency. PWF for duck meat is 26.79, which indicates a denser consistency. All types of raw materials under study have high molding properties, which indicate the possibility of combining them.

A comparison of the functional and technological properties of fish and duck forcemeat confirms that the addition of salt enhances WHC, WBC, and FHC. At the same time, WBC and WHC of fish are higher due to higher water levels, which, combined with low-watering duck meat, can be predicted to create a high-fat forcemeat system. A comparative analysis of the rheological properties of samples allows suggests that the combination of high-viscosity fish will compensate for the low fluidity of Muscovy duck meat.

The ability to emulsify and holding fat in the state of emulsion in minced duck meat has proven to be better. A combination with fish minced meat can compensate for the ability to retain fat in the system of combined products. Thus, due to the basic functional properties of raw materials and chemical composition, regional aquaculture, combined with waterfowl meat, will not only improve the technological properties of combined meat systems but also balance them by correcting the composition of proteins and fats.

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EFFECT OF MODELLED STRESS AND ADAPTOGENS ON MICROSTRUCTURAL CHARACTERISTICS OF PORK FROM FAST-GROWING HYBRID ANIMALS

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ABSTRACT
This research aimed to study the effect of modelled technological stress and the introduction of selenium and dihydroquercetin (DHQ) into pig diets on the microstructure of *M. longissimus dorsi* muscle tissue. The *in vivo* experiment was carried out on 36 hybrid young barrows (Large White x Landrace) x Duroc) with an initial live weight of 34 – 36 kg until they reached a weight of not less than 110 kg. The animals were divided into four groups: 1 (C-) – pigs did not receive adaptogens and were not subjected to modelled technological stress; 2 (C+) – pigs did not receive adaptogens but were subjected to stress via relocation of animals; 3 (C+Se) – pigs were subjected to stress and received 0.2 mg $Se.kg^{-1}$ feed as selenium proteinate in addition to their diet; 4 (C+DHQ) – pigs were subjected to stress and received 32 mg $DHQ.kg^{-1}$ feed in addition to their diet. The best results regarding the muscle tissue condition were recorded in *the musle L. dorsi* samples were taken from the carcasses of group 4 (C+DHQ). Analysis of variance using the Fisher–Snedecor test confirmed that addition of adaptogens led to an improvement of the pH_{24} value (at $p = 0.05$, $f_{observed} = 5.90 > f_{critical} = 4.17$) and moisture-holding capacity (at $p = 0.05$, $f_{observed} = 3.04 > f_{critical} = 2.92$). The effect of long-term addition of DHQ to pig diets (78 days) on the condition of muscle tissue was studied for the first time, which allowed us to conclude its role in the prevention of myopathic changes in the muscle fibre structure.

Keywords: pork; muscle tissue; myopathy; adaptogen; stress

INTRODUCTION
It is known that the development of degenerative (myopathic) changes in the muscle tissue structure which affect pork quality is a consequence of lifetime stress on the animal and/or inadequate nutrition **(Semenova et al., 2019)**. All types of mammals are exposed myopathic changes **(Herraez et al., 2013)**. Pig breeding and poultry farming have the most economic losses due to myopathic. On poultry farms, up to 70% of the poultry stock is affected by myopathy, which manifests itself on the breast muscles **(Kijowski and Kupinska, 2012)**. Hybrid fast-growing pigs are more sensitive to stress. In these animals, nodes, and bands of super contraction (or hyper contraction), the appearance of which leads to deterioration of pork technological and consumer quality, are seen in muscles after slaughter.

Therefore, the aim of this research was to study the effect of modelled technological stress and the introduction of adaptogens (selenium and dihydroquercetin (DHQ)) into pig diets on the muscle tissue microstructure of *M. longissimus dorsi*.

Scientific hypothesis
The muscles of fast-growing hybrid pigs are characterized by signs of myopathy, which develop in conditions of limited animal movement and under the influence of stress. Destructive changes in muscle tissue leading to a decrease in meat quality. Use adaptogens into feed can reduce the severity of signs of myopathy.

MATERIAL AND METHODOLOGY
The experiment was carried out *in vivo* on 36 hybrid young barrows (F-2 hybrids: (Large White x Landrace) x Duroc)) with an initial live weight of 34 to 36 kg. The animals were kept in the physiological yard of the L.K. Ernst Federal Science Center for Animal Husbandry (VIZh) from May 21 to September 28, 2019. Animals in all groups were kept under the same conditions (temperature, humidity and light regimes, the gas composition of the air inside the building), which corresponded to the zoohygienic norms in all groups. Animals were fed according to the norms of VIZh **(Nekrasov, Golovin and Makhaev, 2018)**. The animals were selected according to the pair-analogue principle and divided into four groups with nine animals in each group:

- control group 1 (C-) – pigs did not receive adaptogens and were not subjected to modelled technological stress;
- control group 2 (C+) – pigs did not receive adaptogens but were subjected to stress after the nursery period until slaughter;
- experimental group 3 (C+Se) – pigs were subjected to similar stress and received selenium proteinate in an amount of 20 mg.kg^{-1} feed (or 0.2 mg Se.kg^{-1} feed) additionally to their diet throughout the experiment until slaughter;
- experimental group 4 (C+DHQ) – pigs were subjected to similar stress and received the dihydroquercetin preparation in an amount of 40 mg.kg^{-1} feed (or 32 mg DHQ.kg^{-1} feed) additionally to their diet throughout the experiment until slaughter.

To create a stress factor in groups C+, C+Se and C+DHQ, the animals were kept in three pens with three pigs in each and were subjected to relocation after 14 days, which resulted in new "neighbors" in a pen (Table 1). In control group 1, animals were also kept in pens with three pigs per pen, but they were not relocated. Each pen corresponded to the norms of pig keeping (concrete floor (1.5 × 2.0 m) with a rubber cover (1.5 × 1.0 m) and was equipped with a nipple drinker and a group feeder with two dividers.

The period of pig fattening consisted of two stages with a diet corresponding to the physiologicalrequirements of animals. The duration of the stages were 42 and 36 days, respectively. Animals were slaughtered using the so-called "peasant method" after they reached a live weight of not less than 110 kg under the conditions of the physiological yard. The pigs were immobilized with a powder-charged handheld captive bolt stunner with a shortened bolt. Samples of muscle tissue were taken from the *M. longissimus dorsi* of chilled carcasses at the level of the last thoracic vertebra and the 6th and 7th lumbar vertebrae.

The following methods were used in the study:

- live weight and weight of hot carcasses were measured by weighing each animal (hot carcass) on REUS-A-PK brand and an error of weight measurement (scale interval) of 0.1 kg; weighing scales with a possible load range of 0 to 300; weighing scales with a possible load range of 0 to 300 kg;
- backfat thickness was measured using a ruler according to **GOST 427-75 (1977)** by measuring backfat thickness over spinous processes between the 6th and 7th thoracic vertebrae without counting hide thickness;
- carcass category was determined depending on carcass weight and backfat thickness according to **GOST 31476 (2012)**;
- pH was measured by the potentiometric method using a pH meter "Teslo 205" with a measurement error of ±0.02. Chilled carcasses were sorted by the mean pH value obtained after statistical processing of the results of not less than five parallel measurements in *M. longissimus dorsi*;
- moisture-holding capacity (MHC) was determined using the press method by Grau and Hamm with the modifications of Volovinskaya **(Zhuravskaya, Alekhina and Otryashnikova, 1985)**;
- microstructural investigations were carried out according to **GOST 19496-93 (1995)**; muscle tissue types were determined using Sudan black staining.

The research was approved by the bioethical commission of the V.M. Gorbatov Federal Research Center for Food Systems of the Russian Academy of Sciences (protocol #03/2019, dated May 31, 2019).

Statistical analysis

Statistical analysis was carried out by least-squares mean comparisons using the PDIFF option of the general linear model procedure (SAS, 2002; SAS Inst. Inc., Cary, NC, USA). Each pig was considered as an experimental unit in measuring growth performance, while an individual sample of pork muscle tissue was used as an experimental unit for analyzing functional technological indices and ratio of different muscle fibre types in *M. longissimus dorsi*. The differences in MHC between groups were confirmed statistically using the Fisher–Snedecor test (F-distribution at $p = 0.05$, degrees of freedom: 3 and 31; $f_{observed} = 3.04$, $f_{critical} = 2.92 \Rightarrow f_{observed} > f_{critical}$). Statistical differences were considered highly significant at $p < 0.01$, significant at $p < 0.05$ and as a tendency between $p \geq 0.05$ and $p \leq 0.10$.). The obtained experimental data were processed biometrically with STATISTICA (vers. 10, StatSoft, Inc., 2011).

RESULTS AND DISCUSSION

During fattening, all four pig groups showed good results expressed as intensive growth; therefore, animal groups did not display statistically significant differences in live weight at any fattening stage nor in hot carcass weight (Table 2).

The fact that live animal weight and carcass weight in all four groups, including the animals that received adaptogens, did not show significant differences and all carcasses were assigned to the 2nd category is in good agreement with the results of foreign studies from different years **(Mahan, Cline and Richert, 1999; Ivanova et al., 2019)**.

Table 1 A scheme of animal relocation to create a stress factor by the example of one group (control group).

Date	Subgroup 2.1.			Subgroup 2.2.			Subgroup 2.3.		
02.07.19	1	2	3	4	5	6	7	8	9
16.07.19	1	4	7	2	5	8	3	6	9
30.07.19	3	5	7	1	6	9	2	4	8
13.08.19	2	6	9	3	4	7	1	5	8
27.08.19	1	4	9	2	5	8	3	6	7

Note: (M ±m, n = 9).

Table 2 Live weight of the experimental pigs, weight of hot carcasses and backfat thickness in the control and experimental groups.

Item	Mean value for groups			
	Control group 1, C-	Control group 2, C+	Experimental group 3, C+Se	Experimental group 4, C+DHQ
Live weight at the beginning of fattening, kg	34.71 ±1.24	34.64 ±1.68	34.89 ±0.96	34.51 ±1.11
Live weight at the end of the 1st period, kg	74.33 ±1.85	74.90 ±2.45	73.59 ±0.98	74.43 ±1.85
Live weight at the end of fattening, kg	116.19 ±1.83	115.42 ±1.51	113.03 ±0.56	115.77 ±1.39
Live weight after fasting, kg	109.86 ±1.54	109.76 ±1.36	106.90 ±0.90	109.61 ±1.34
Hot carcass weight, kg	80.04 ±1.48	78.31 ±2.68	78.71 ±0.84	79.91 ±1.09
Backfat thickness, mm	20.44 ±1.45	20.78 ±0.20	20.44 ±1.40	21.33 ±2.05

Note: * – without head, legs, tail, internal organs and internal fat, ** – between the 6th and 7th thoracic vertebrae of the left half-carcass without hide thickness.

Uniformity of the studied animals by weight was important for the following investigation of the effect of stress and adaptogens on muscle tissue microstructure. For example, when studying myopathy in fast-growing chickens and turkeys, it was established that poultry weight affected the frequency of manifestation of pathological changes in muscle tissue **(Kijowski and Konstanczak, 2009)**. In experiments on pigs, not only animal carcass weight but also the backfat thickness and the content of muscle tissue in a carcass (leanness) were assigned to risk factors **(Minvielle et al., 2001)**.

Moreover, non-uniformity of hot carcasses by weight and leanness leads to problems with their chilling after slaughter, which, in turn, can be reflected in post-mortem formation of muscle tissue microstructure **(Petracci and Cavani, 2012)**.

Therefore, significant differences in animal weight and leanness were undesirable as they could influence the development of myopathic changes in muscle tissue, "blurring" the picture of an effect of technological stress and adaptogens. It was noted in our experiment that control group 2 (C+) showed the lowest value of hot carcass weight, and experimental group 3 (C+Se) was distinguished by the lowest values of live weight beginning from the end of the 1st period of fattening up to slaughter. However, firstly, these differences in groups were not statistically significant; secondly, all carcasses obtained as a result of the slaughter of pigs from the control and experimental groups were assigned by weight and backfat thickness to the second category according to the existing Russian standard **(GOST 31476, 2012)**. It was noted in our experiment that control group 2 (C+) showed the lowest value of hot carcass weight, and experimental group 3 (C+Se) was distinguished by the lowest values of live weight beginning from the end of the 1st period of fattening up to slaughter. However, firstly, these differences in groups were not statistically significant; secondly, all carcasses obtained as a result of the slaughter of pigs from the control and experimental groups were assigned by weight and backfat thickness to the second category according to the existing Russian standard **(GOST 31476, 2012)**. The frequency of myopathy manifestation can be linked to reduced pH values in chilled pork. However, many researchers paid attention to the fact that there is a difference between meat with the signs of PSE and meat with the signs of myopathy **(Minvielle et al., 2001; Minvielle et al., 2001)**. The obtained data suggest that the carcasses from groups 1, 2, 3, and 4 did not display statistically significant differences in the average pH_{24} value (Table 3). Moreover, analysis of variance using the Fisher–Snedecor test (F-distribution at $p = 0.05$, degrees of freedom: 3 and 35) confirmed the invalidity of the hypothesis about the presence of differences in the control and experimental groups by the pH_{24} value ($f_{observed} = 2.00$, $f_{critical} = 2.92 \Rightarrow f_{observed} < f_{кр}$).

Quite low pH_{24} values (5.41, 5.40, 5.49 and 5.47) in groups 1, 2, 3, and 4, respectively, were explained by stress in animals directly at slaughter (peculiarities of "peasant slaughter") and indicated distinct manifestation of PSE properties in all meat samples. With that, a trend towards less pronounced PSE properties was observed in the experimental groups that received adaptogens compared with the control. Verification of this statistical hypothesis (at $p = 0.05$, degrees of freedom: 1 and 35) showed its validity ($f_{observed} = 5.90, f_{critical} = 4.17 \Rightarrow f_{observed} > f_{critical}$). This conclusion is in agreement with the results of many studies, which found a positive effect of organic selenium **(Mahan, Cline, and Richert, 1999; Mateo et al., 2007; Li et al., 2011; Calvo et al., 2016; Calvo et al., 2017; Jiang et al., 2017)** and DHQ **(Vlahova-Vangelova et al., 2019)** on the ultimate pH value of pork. The mean values of MHC for control groups 1 and 2, and experimental groups 3 and 4 were 62.61%, 67.05%, 69.18%, and 69.49%, respectively (Table 3). The differences in MHC between groups were confirmed statistically using the Fisher–Snedecor test (F-distribution at $p = 0.05$, degrees of freedom: 3 and 31; $f_{observed} = 3.04, f_{critical} = 2.92 \Rightarrow f_{observed} > f_{critical}$). Thus, the samples from all four groups were different regarding functional-technological properties in the absence of significant differences in muscle tissue pH in chilled pork.

The obtained results showed that the modelled technological stress during animal fattening and the use of adaptogens positively affected pork MHC. Similar conclusions regarding adaptogens were drawn by other studies **(Mahan, Cline, and Richert, 1999; Mateo et al., 2007; Li et al., 2011; Calvo et al., 2016; Calvo et al., 2017; Jiang et al., 2017; Vlahova-Vangelova et al., 2019; Kremer, Stahly and Sebranek, 1998)**.

Differences in MHC in the absence of differences in pH_{24} could be associated with different ratios of the main muscle fibre types in pork.

Table 3 Functional technological indices of pork muscle tissue.

Parameters	Values of the indices for groups			
	Control group 1, C-	Control group 2, C+	Experimental group 3, C+Se	Experimental group 4, C+DHQ
pH_{24} value*	5.41 ±0.06	5.40 ±0.08	5.49 ±0.14	5.47 ±0.09
MHC**, %	62.61 ±3.54	67.05 ±4.79	69.18 ±4.90	69.49 ±6.82

Note:* - (M ±m, n = 9); ** - (M ±m, n = 8).

Table 4 Ratio of different muscle fibre types in *M. longissimus dorsi*.

Item	Proportion of muscle fibres of the corresponding type, % of total amount / % of maximum value			
	Control group 1, C-	Control group 2, C+	Experimental group 3, C+Se	Experimental group 4, C+DHQ
White (II type)	78.4 ±0.8/99.7	78.6 ±0.8/100.0	78.5 ±0.8/99.9	78.6 ±0.8/100.0
Red (I type)	10.5 ±0.1/88.2	10.2 ±0.1/85.7	10.8 ±0.1/90.8	11.9 ±0.1/100.0
Intermediate types	11.1 ±0.1/99.1	11.2 ±0.1/100.0	10.7 ±0.1/95.5	10.2 ±0.1/91.1

Note: (M ±m, n = 5)

Table 5 Results of microstructural investigations of muscle tissue samples from *M. longissimus dorsi*).

Item	Control group 1, C-	Control group 2, C+	Experimental group 3, C+Se	Experimental group 4, C+DHQ
Morphometrical characteristics of muscle tissue				
Min sarcomere length, µm	1.4 – 1.8	1.7 – 2.1	1.5 – 2.0	1.5 – 1.9
Max sarcomere length, µm	1.7 – 2.0	1.9 – 2.3	1.7 – 2.5	2.0
Min perimysium thickness, µm	10.0	20.0	20.0	20.0
Max perimysium thickness, µm	20.0	50.0	50.0 – 55.0	35.0 – 40.0
Min thickness of fat tissue layers, µm	40.0 – 70.0	50.0 – 70.0	60.0 – 70.0	50.0 – 70.0
Max thickness of fat tissue layers, µm	130.0 – 200.0	170.0 – 200.0	200.0 – 330.0	200.0 – 240.0
Mean diameter of muscle fibres, µm	44.5 – 48.6	45.2 – 47.8	45.7 – 48.5	45.8 – 47.3
Signs of myopathy in white muscle fibres				
Min fibre diameter in nodes of contraction, µm	60.0	80.0	80.0	80.0
Max fibre diameter in nodes of contraction, µm	80.0	100.0	100.0	100.0
Max length of the supercontraction band, µm	800.0	400.0	800.0	300.0 – 350.0
General conclusion				
Pathological changes	Myopathy	Moderate myopathy	Myopathy	Moderate myopathy

Note: (n = 5)

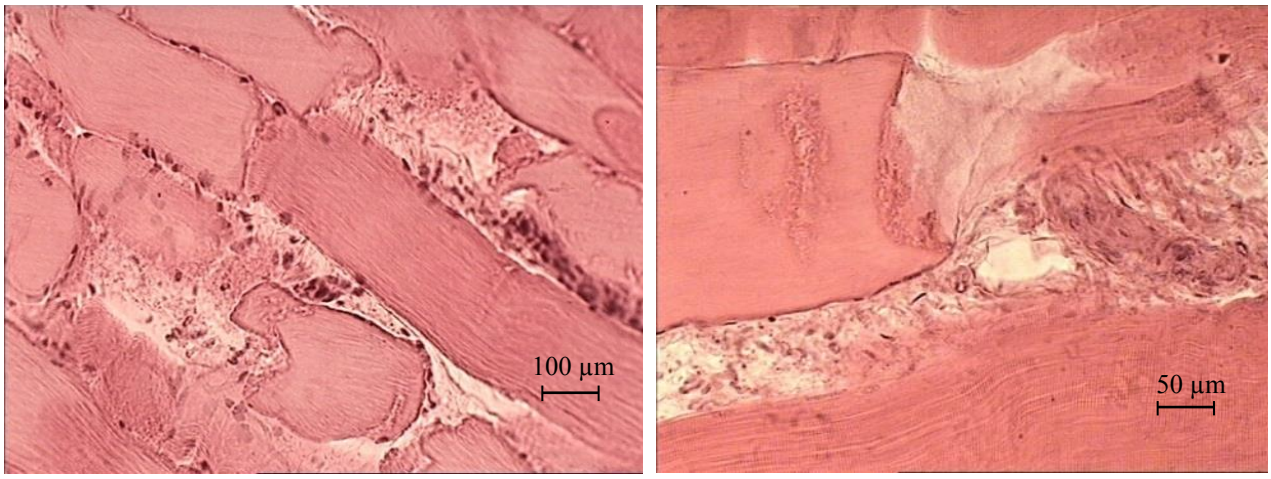
Muscle fibres are dynamic structures, which can change from one type to another under certain conditions **(Listrat et al., 2016)**.

The proportions of type I fibres (red, slow, oxidative), type II fibres (white, fast, glycolytic) and intermediate types depends on many factors, including muscle function, animal species, breed, age, physical activity, ambient temperature, nutritional and so on.

It is known that porcine skeletal muscles, especially in fast-growing hybrids, show a tendency towards an increased content of type II muscle fibres **(Lefaucheur et al., 1998)**. With that, several studies show that destructive changes in muscle tissue, including the development of myopathy and meat quality deterioration, are linked with the prevalence of type II fibres **(Realini et al., 2013; Kim et al., 2018; Lee et al., 2012)**. The highest content of type II fibres is typical for *M. longissimus dorsi*. In this context, it is recommended that this muscle be sent for histological investigations to diagnose myopathic conditions in pigs **(Realini et al., 2013; Cooper and Valentine, 2015)**.

The proportion of type II (glycolytic) fibres influences a tendency in meat tissue towards post-mortem development of nodes and bands of hypercontraction (so-called "giant fibres"), which are formed during the pig's lifetime and directly linked with poor quality meat **(Kim et al., 2018; Schubert-Schoppmeyer et al., 2008)**.

In our experiment, microstructural investigations of *M. l. dorsi* (Table 4) showed that the ratio of the main muscle fibre types was within the range of literature data (78.4% to 78.6%). According to the results of similar studies **(Listrat et al., 2016; Sales and Kotrba, 2013)**, the proportion of white fibres in *m. L. dorsi* of fast-growing hybrids can be up to 90%. As a rule, red and intermediate fibres on average account for 10% each. Our results are in agreement with these tendencies.



a) b)

Figure 1 Microstructure of muscle tissue with signs of myopathy. Note: Longitudinal section. Non-uniform cross-striation (samples from groups 1 and 3). a) – nodes and bands of supercontraction with ruptures of myofibrils with a length of up to 800 µm. b) – formation of fine-grained protein mass at the sites of muscle tissue destruction as a result of damage to the sarcolemma integrity.

As follows from the data in table 4, some differences in the content of red and intermediate fibres were observed between the samples from the control and experimental groups. For example, the samples from experimental group 4 were characterized by the highest content of red fibres (11.9%) and the lowest content of intermediate fibres (10.2%), which likely had a positive effect on the functional-technological characteristics of muscle tissue but do not fully explain them.

During microstructural investigations of sections stained with haematoxylin and eosin, the complex of morphometrical indices of muscle tissue was determined, including the characteristics of white muscle fibres (Table 5).

Minimum and maximum sarcomere length in samples of *m. Longissimus dorsi* from animals of all groups were characterized by a rather wide range of values. The most stable sarcomere sizes were observed in sections of the samples from group 4. All samples from carcasses of animals subjected to the stress factor (groups 2, 3 and 4) showed a tendency towards an increase in the thickness of the connective tissue layers (perimysium) and an increase in the thickness of the fat tissue layers. The latter tendency was most pronounced in the muscle tissue samples from animal carcasses of groups 3 and 4, which received adaptogens. The mean diameter of muscle fibres without signs of supercontraction in the samples of all groups was quite uniform and corresponded to the animal species and characteristics of the muscle that was chosen for investigation (*M. longissimus dorsi*).

At the same time, all samples showed signs of myopathy, which were manifested in peculiarities of generative changes in the structure of white muscle fibres. In the samples of control group 1, the diameter of nodes of contraction in the cross-sections was 60 to 80 µm. The bands of super contraction with multiple ruptures of myofibrils with a length of up to 800 µm with non-uniform cross-striation were observed in the longitudinal sections. The fine-grained protein mass was found at the sites of muscle fibre destruction as a result of damage to the sarcolemma integrity.

Similar changes in the structure of white muscle fibres were also observed in the muscle tissue samples from the third group of animals, which received selenium. With that, the fibre diameter in the nodes of contraction was 80 to 100 µm.

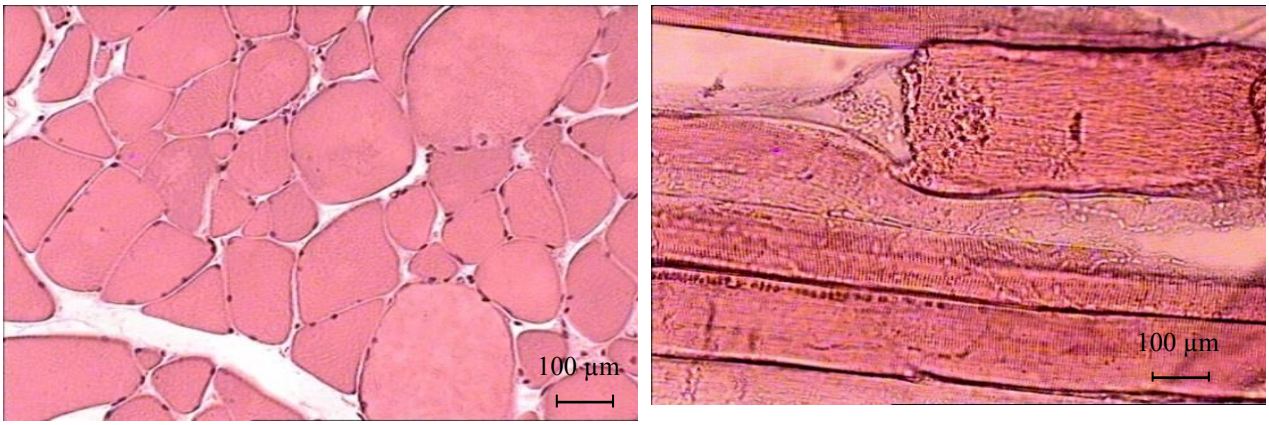
The revealed pathological changes in the samples from groups 1 and 3 corresponded to the picture of myopathy (Figure 1 and Figure 2).

In the samples from group 2, upon similar sizes of white fibres in nodes of contraction (80 to 100 µm), the bands of super contraction with the lengths of not more than 400 µm were observed in the longitudinal sections, which allowed diagnosis of only moderate myopathy.

In the samples from group 4, bands of super contraction had a maximum length of not more than 350 µm, which undoubtedly facilitated better retention of pork functional-technological properties and partly explained the high results obtained in MHC measurement. On the other hand, a higher proportion of red fibres was found in the samples from group 4, which can also facilitate higher MHC values. The pathological changes revealed in the samples from groups 2 and 4 corresponded to the picture of moderate myopathy (Figure 3).

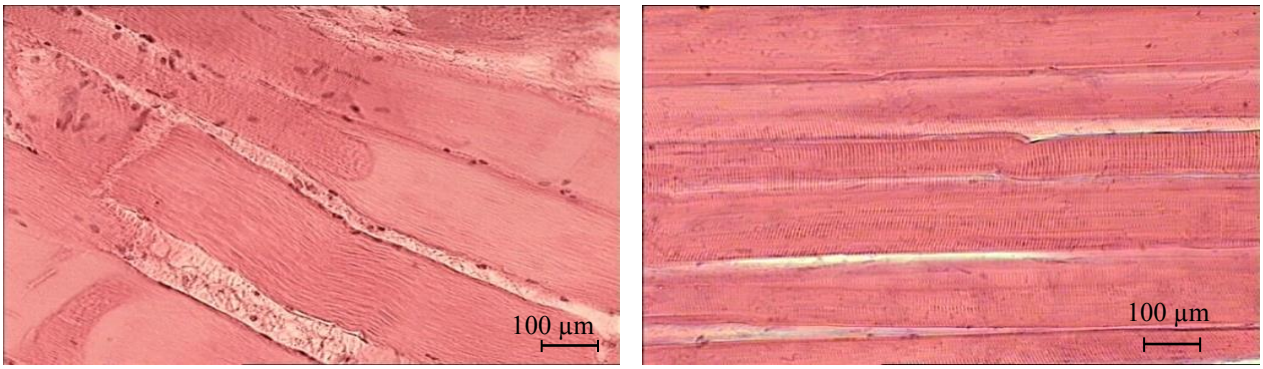
A comparison of the results of microstructural investigations showed that supplementation of animal diets with DHQ had a greater effect on the condition of pork muscle tissue than the use of selenium in the diet. These results are in good agreement with the data of other researchers.

For example, it was concluded in three studies **(Calvo et al., 2016; Falk et al., 2018)** that the addition of selenium, irrespective of the source, in the diet did not influence the muscle tissue condition. However, it was suggested that during meat aging selenium could enhance destructive changes in muscle tissue caused by an increase in the activity of tissue enzymes. It is necessary to note that data on the effect of DHQ on the condition of pork muscle tissue w not found in the available scientific literature.



a) b)

Figure 2 Microstructure of muscle tissue with signs of myopathy (samples from groups 1 and 3). Note: a) – nodes of supercontraction with the round shape in the cross section and a diameter more 100 µm; b) – nodes of supercontraction of myofibrils with pronounced longitudinal striation and ruptures of the sarcolemma more 100 µm in the longitudinal section.



a) b)

Figure 3 Microstructure of muscle tissue with signs of moderate myopathy (samples from groups 2 and 4). Longitudinal section. Note: a) – nodes of supercontraction of myofibrils with ruptures of sarcolemma and pronounced longitudinal striation with a length near 400 µm. Non-uniform cross-striation; b) – transversal bands of myofibril supercontraction in the muscle fibre structure Muscle fibres with the straight shape and pronounced cross-striation lay loosely in relation to each other.

However, the results of recent studies on laboratory animals have shown that long-term addition of quercetin (analogue of DHQ) reduces muscle damage **(Hollinger et al., 2015; Mukai et al., 2016).**

Therefore, the combination of modelled technological stress during animal fattening with the addition of DHQ into a diet partly leveled pork quality deterioration caused by an effect of pre-slaughter stress in animals, allowing the production of meat with moderate signs of myopathy and, consequently, with higher functional-technological characteristics.

CONCLUSION

A decrease in pork functional-technological characteristics, such as MHC, is not always conditioned to the full extent by the pH value of muscle tissue. The manifestation of myopathy, which is expressed in destructive changes in muscle tissue, is typical for fast-growing hybrid animals fattened under conditions of industrial enterprises. The results of this study showed that the presence of such modelled technological stress factors as the relocation of animals during fattening positively influenced the muscle tissue microstructure after slaughter. At the same time, additional incorporation of DHQ as an adaptogen reduced the development of myopathy signs to a greater extent. DHQ showed better results in this regard than selenium as a distinct manifestation of destructive regions in muscle tissues was lower in the group of animals that received DHQ in the diet for a long time. In the future, accumulation and systemization of scientific data on the manifestation of pathological changes in the muscle tissue structure and methods for their prevention will allow more effective management of the process of lifetime formation of pork quality.

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IDENTIFICATION OF THE NEEDS OF PRIMARY MILK PRODUCERS IN THE NEW COMMON AGRICULTURAL POLICY 2021 – 2027

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ABSTRACT

Although Slovakia belongs to the smallest milk producers in the EU, we have to bear in mind the importance of the milk industry for the economy of Slovakia. Current expectations in Slovakia are that primary milk producers are able to produce milk at the lowest possible price, with state support on the same level as in other EU states, processing companies have a good purchasing price for the raw materials, retailers are able to share margins and to increase the market share of Slovak dairy products and last but not least that consumers buy milk and dairy products produced in Slovakia. Milk production in primary production has a profound ecological and social benefit for our countryside but the most important is the contribution of milk and dairy products to human health. The Slovak dairy sector has experienced three dairy crises and one butter crisis in the last ten years, what caused that up to 37% of milk producers in Slovakia cease their production. Currently, there are 401 approved primary milk producers in Slovakia. This sector is in long-term condition weakened by market fluctuation and by previous crises. Slovakia has a large investment debt in this sector. That is why in order of food security and maintaining employment it should be in the interest of the new government to do everything possible to stabilize this sector. Therefore, the primary objective of this scientific paper is to identify the needs of primary milk producers in Slovakia in relation to the strategic objective – to increase the competitiveness of the milk producers. The research aims to transform the voice of primary milk producers into the new programming documents for the upcoming period of the Common Agricultural Policy (CAP) for the years 2021 – 2027.

Keywords: milk; primary milk producers; dairy sector; common agricultural policy

INTRODUCTION

The importance of milk and dairy products to human nutrition has been repeatedly confirmed by nutritionists around the world. Milk, as the most complex food, has been one of the main foods since ancient times. The existence of human society cannot be imagined without the milk. Its composition makes milk irreplaceable for a healthy diet.

Milk is a highly nutritious food containing many macro- and micronutrients that are essential for the growth and maintenance of human health **(Iqbal et al., 2015)**. The main biological components of milk include protein, fat, lactose, minerals (calcium, phosphorus, potassium, magnesium) as well as essential nutritional substances, vitamins and other important substances, which form a comprehensive balanced food for all age categories of the population **(De la Fuente and Juárez, 2015; Šimo, Mura, and Buleca, 2016)**.

Milk proteins present in the milk are made of casein and whey proteins which are nutritionally and technologically essential and represent an irreplaceable amino acid intake for humans **(Bartošovičová, 2011)**. Milk proteins contain 18 out of 22 essential amino acids that cannot be created by the human body itself and that are inevitable for running of the human body; and are divided into two basic groups, i.e. caseins and whey proteins. Milk is rich in vitamins, namely vitamin A, B (especially B1, B2, B3, B6, and B12), C, D, E, and K **(Gonda, 2009)**.

Other essential nutrition components of milk are carbohydrates (especially lactose) and fats. Lactose is a hydrocarbon that can only be found in milk and has a significant role in energy production. Lactose is simply a certain kind of sugar presented in milk **(Kurajdova and Táborecká-Petrovičová, 2015)**. Milk fat, representing an easily digestible emulsion containing lecithin and a relatively low level of cholesterol, is another important ingredient in milk **(Kubicová, Predanocyová and Kádeková, 2019)**.

Besides the fact that milk has a notable positive nutritional impact on the human body, it is characterized by a pleasurable effect on health and disease prevention. Although a lot of various researches and studies on health aspects and disease prevention effects of drinking milk and consuming milk products are still in progress, there are

several interesting conclusions and findings which came to light.

The most recent scientific evidence supports the consumption of cow's milk and dairy products as part of a balanced diet. Current scientific literature suggests that appropriate consumption of milk and its derivatives may be beneficial at all ages, except specific medical conditions such as lactose intolerance or milk protein allergy **(Marangoni et al., 2019)**.

Scientific hypothesis

This research paper aims to identify the needs of primary milk producers in Slovakia in relation to two strategic objectives (to increase the competitiveness of the milk producers and their position in value chains) and transform their voice into the new programming documents for the upcoming period of the Common Agricultural Policy for the years 2021 – 2027.

Several hypotheses were formulated:

Hypothesis 1: There exists a dependence between the legal form of business and land area.

Hypothesis 2: There exists a dependence between the legal form of business and the number of dairy cows.

Hypothesis 3: There exists a dependence between the legal form of business and number of employees.

Hypothesis 4: There exists a dependence between the region and average milk yield per cow.

MATERIAL AND METHODOLOGY

In order to fulfill the stated objectives, we chose a questionnaire survey as the main method. This method was chosen mainly because the questionnaire generally represents a series of different types of questions that are answered by respondents. The content, number, and type of questions are determined by the purpose of the survey and the target group for which the questionnaire is intended. The questionnaire is suitable for obtaining data from many people. Due to the uniformity of the formulations, the data can be processed, and the responses of different groups can be easily compared. In general, the questionnaire is an "economical" tool in terms of data collection and processing (depending on the type of questions).

The questionnaire survey aimed to gather information from primary milk producers, to analyse the current situation and identify the needs of primary milk producers in the new CAP 2021 – 2027. For this purpose, the questionnaire consisted of two parts:

1) The current state of the primary milk producers.
2) Identification of the needs in the new CAP 2021 – 2027 concerning the strategic objective – to increase the competitiveness of the milk producers

The survey was carried out in September – November 2019 by sending a link to an electronic questionnaire by e-mail but mainly by personal inquiry of the Slovak primary milk producers.

The criteria for determining the survey sample were all primary milk producers in Slovakia. Out of a total of 401 primary milk producers in Slovakia, we managed to reach 104 primary milk producers, who represent 27% of the total number of respondents surveyed. In Figure 1 below, we can see the legal form of the respondents surveyed.

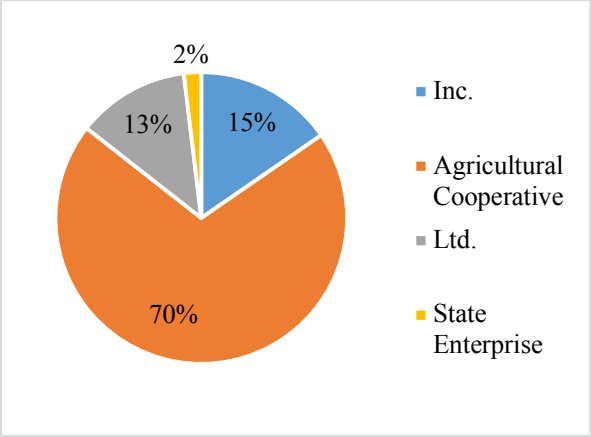

Figure 1 The legal form of the respondents surveyed. Note: Own processing based on questionnaire survey.

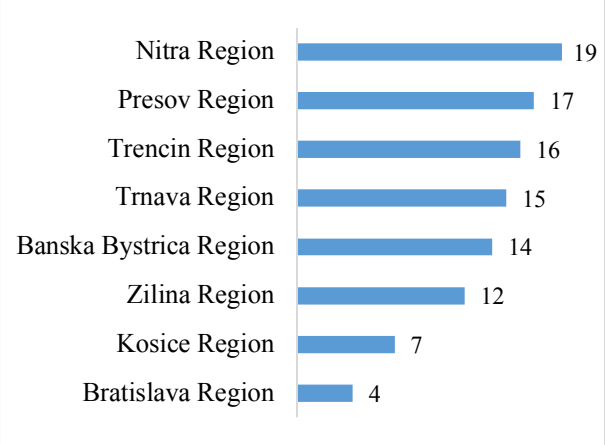

Figure 2 Regional representation of enterprises. Note: Own processing based on questionnaire survey.

Table 1 Number of dairy cows as of 31/08/2019 in Slovakia.

Indicator	Total SR (pc)	Analyzed (pc)	Share in SR (%)
Number of dairy cows as of (31/08/2019)	124 030	40 713	33%

Note: Own processing based on Statistical Office of Slovak Republic.

The largest share of respondents was represented by agricultural cooperatives (70%).

Figure 2 shows the regional representation of the respondents surveyed. The largest share of respondents was from the Nitra region.

In Table **1** below the number of dairy cows can be seen. In Slovakia, there are registered in a total of 124 030 dairy cows as of 31/08/2019. Together our analysed sample had 82 303 all cattle categories and out of this number, our analysed sample had in total 40 713 dairy cows which represent 33% share in Slovakia. The smallest enterprise had just 103 dairy cows and the largest enterprise had 3 146 dairy cows.

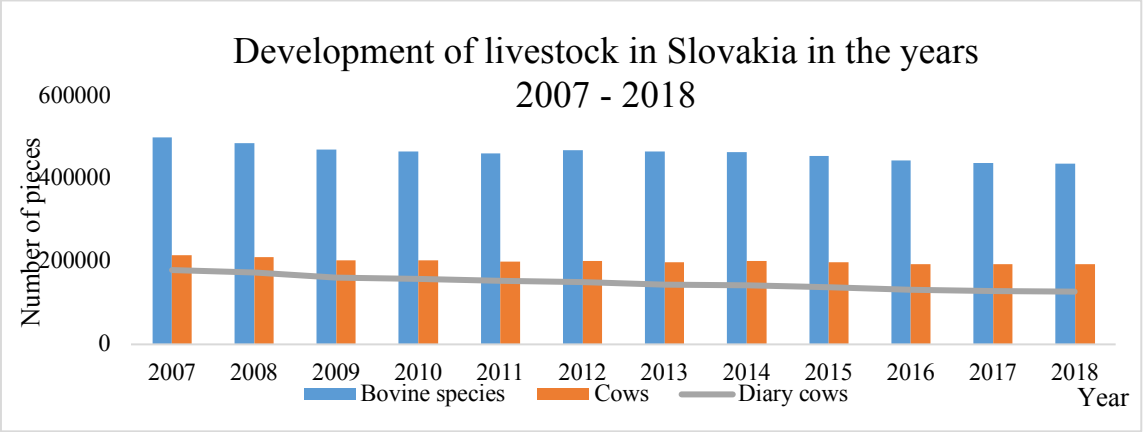

Figure 3 Development of livestock in Slovakia in the years 2007-2018. Note: Own processing based on Statistical Office of Slovak Republic.

Table 2 Development of the Consumption of Milk and Dairy Products in kg per capita in the SR (2007 – 2017).

	Year										
	2007	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**
Milk and dairy products (kg)	153.4	153.0	153.8	162.8	156.9	158.6	158.5	166.8	169,2	176,2	174,6
Milk (kg)	52.4	48.3	49.5	54.5	53.1	54.3	49.3	48.3	48.1	46.5	46.4

Note: Own processing based on Statistical Office of Slovak Republic.

After analysing the number of dairy cows we also decided to analyse milk deliveries of our analysed sample.
In Table 2 the share of analysed enterprises in the milk deliveries in Slovakia can be seen. In our survey, we analysed 40% of the whole milk deliveries in the Slovak Republic.

Statistical analysis

The selection of tests is designed particularly because of variables in research. The attention is paid to the formulation of hypotheses (null and alternative) and to determine the level of significance. The collected data were processed by using Microsoft Excel and subsequently evaluated in the statistical program XL Stat. The formulated hypotheses were tested by applying the following statistical tests:

1) Shapiro–Wilk test
2) Chi-square contingency test
3) ANOVA test
4) Kruskal Wallis test

In hypothesis testing, if the p-value is lower than a significant level, in the case of XL Stat software by Addinsoft (version 2019.3.2), it is 0.05, the null hypothesis was rejected and the alternative hypothesis was confirmed.

RESULTS AND DISCUSSION

Raw cow milk represents one of the most important commodities in the agricultural market. The Slovak agro-food foreign trade is characterized by a substantial increase in the commodity trade, monitoring of competitiveness is therefore very important **(Šimo, Mura, and Buleca, 2016)**. The major part of the milk production is represented by the cow milk. Other milk types such as the sheep, goat, and milk of other species have specific properties as well as the use in human and animal consumption **(Matić et al., 2014; Gavurová et al., 2014)**.

In Slovakia, there are currently approved 401 primary milk producers that process milk. The Slovak dairy sector has experienced three dairy crises in the last ten years. The Slovak dairy sector is weakened and financially undersized due to large fluctuations in the market. In the interests of food security and maintaining employment, it is important to do everything possible to stabilize the dairy sector.

Cows' breeding can be considered as strategic, especially concerning other categories of cattle and its connectivity to arable land and permanent grass-land. Cattle breeding represents a crucial condition to maintain a balance between the plant production and breeding processes of agricultural business activities **(Siničáková, 2012)**.

In the reporting period, we can observe a decline in livestock in Slovakia (Figure 3). Likewise, the decreasing character can be observed in the number of cows together as well as in the number of dairy cows. It is interesting to note that on the 1st April 2015, when milk quotas were abolished, the number of cows in Slovakia did not increase. However, in recent years Slovakia has had an annual milk production quota of 1 billion litters, which has not been fulfilled.

Table 3 Milk deliveries for the year 2018 (kg) of the analyzed sample.

Indicator	**Total SR (kg)**	**Total Analysis (kg)**	**Share in SR (%)**
Milk deliveries	817 000 000	326 352 835	40%

Note: Own processing based on Statistical Office of Slovak Republic.

The decline in livestock in Slovakia may also be due to a decline in the reproduction and reproductive characteristics of cattle (the indicators of reproduction of the basic herd and reproductive characteristics of cattle decline year-on-year).

By applying the Chi-square contingency test, hypothesis 2 was confirmed that there exists a statistically significant dependency between the legal form of business and the number of dairy cows (p = 0.05). The testing criteria F is higher than the table value χ^2, therefore we reject the null hypothesis.

In the last year in the reported period, the percentage of heifers and cows fertilized and the proportion of heifers transferred to cows decreased.

On the other hand, the percentage of cows culled as a result of cow mortality has decreased. Deaths of calves were slightly increasing year-on-year. Similarly, fertility indicators deteriorated year-on-year. The number of calves born per 100 cows and the number of calves raised per 100 cows decreased by up to 2.71. The average daily weight gain of fattening cattle increased year-on-year by 0.019 kg/KD, reaching 0.765 kg per head per day per year.

In general, the dairy sector is very labour intensive and people are not interested in working there. According to our analysis, on average one employee takes care of 48 dairy cattle. Hypothesis 3 assumed dependency between the legal form of business and the number of employees. By applying the Chi-square contingency test, hypothesis 3 was not confirmed and there is no statistically significant dependency between the legal form of business and the number of employees (p = 0.05). This means that we do not reject the null hypothesis.

Milk, as one category of dairy products, belongs to the group of basic daily-consumed products characterized by relatively high purchase frequency **(Kurajdova, Táborecká-Petrovičová, 2015)**. Based on their nutritional values, milk and dairy products have an irreplaceable role in daily consumption. On the other hand, the consumption of milk and dairy products is experiencing a certain negative trend in Slovakia compared to other countries in Central Europe.

In Table 3, we can see the development of milk and dairy products consumption in 2007 – 2017 (per capita/year). The consumption of milk and dairy products in Slovakia is increasing from year to year but we are still below the recommended consumption by the World Health Organization (WHO).

According to the WHO, the recommended consumption of milk and dairy products is at least 220 kg per person per year. However, the actual average consumption in Slovakia is currently significantly lower. On average it is only 178 kg per person per year. The average consumption in Europe is over 320 kg per person per year. This means that according to the WHO recommendation, each person should consume at least a glass of milk or a cup of sour milk products such as yogurt or acidophilic milk, plus a portion of butter and 100 g of cheese or cheese products daily.

Even though Slovakia belongs to the smallest milk producers in the EU, we have to keep in mind the importance of the milk industry for the economy of Slovakia. Application of the Chi-square contingency test, as well the hypothesis 1 was not confirmed and there is no statistically significant dependency between the legal form of business and land area (p = 0.05). This means, that we do not reject the null hypothesis.

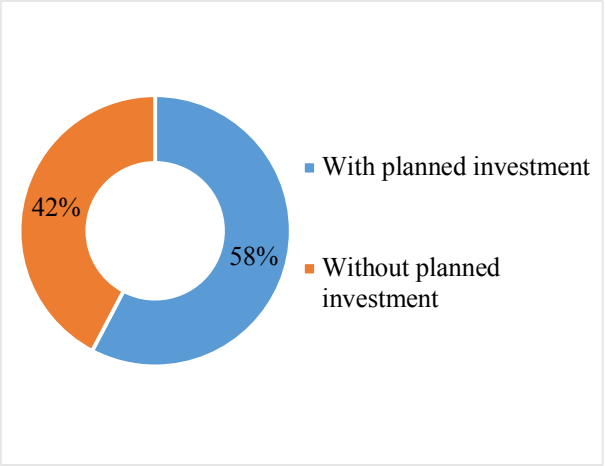

Figure 4 Investments into the housing. Note: Own processing based on questionnaire survey.

The current situation in Slovakia is complicated. We expect:

1) milk producers to be able to produce milk at the lowest possible price,
2) state support at the same level as in other EU states,
3) processing companies to have a good purchase price for the raw material,
4) retailers to be able to share margins and to increase the market share of Slovak dairy products, and
5) consumers to buy milk and dairy products originating in Slovakia.

One of the most important issues within the CAP is the support of milk production **(Folmer, et al., 2013)**. The present experience with the CAP that was introduced into the milk market did not result in the anticipated effects. The price of milk is highly volatile, and the production is changing. The demand for milk has been changing with people consuming less milk per capita worldwide. The success of any future policy affecting the milk market depends on communication between policy makers and farmers **(Alpmann and Bitsch, 2017)**.

Dairy farmers need to invest in keeping their farms in good condition, maintain competitiveness on the market, increase the rate of technology adoption, and improve labour productivity **(Pouch and Trouvé, 2018)**.

For this reason, we decided to identify the needs of primary milk producers and transform their voice in the new CAP 2021 – 2027. Investments realized in dairy farms allow the implementation of new technologies and involve benefits associated with an increase in efficiency, a reduction in costs, an improvement in the quality of products and a reduction in the adverse impact on the environment, and an improvement in animal welfare **(Bewley, 2010)**.

Based on this, in this paper, we focused on analysing the investments of dairy farmers into housing, milking, feeding, water feeding and water supply, storage capacity, pasture system, waste management, and welfare. Firstly, we analysed the investments into the housing – objects for cattle. In Figure 4 it can be seen that 58% of respondents plan the investments into the housing of cattle in the next year. These investments were divided into two main

categories, the reconstruction of the old objects and the construction of the new ones.

Up to 68% of respondents are planning the reconstruction of existing buildings and just 32% of respondents plan to build a new stable for cattle.

Respondents with planning investments into the reconstruction mostly plan the complete reconstruction of the building such as the reconstruction of the roof, electricity, water, replacement of windows, reconstruction of sanitary facilities, isolation), some of them plan just small construction work such as demolition of the side walls to increase the volume of fresh air, to build ventilation slots or to install or replace stall ventilation. In terms of animal housing responders plan to replace fences, bed mattresses, drinkers.

As shown in Figure 5 above, our respondents plan housing in total for 31 545 different categories of bovine species in 2020. These animals are planned for both stables, for the new ones as well as for the reconstructed ones. The highest number of planned animals is represented by cows (17 700 pc.) followed by heifers (5 830 pc.), young cattle (4 850 pc.), and the smallest number is represented by calves (3 165 pc.).

The planned volume of investment for housing by our respondents is approx. 75 million €. Our estimated volume

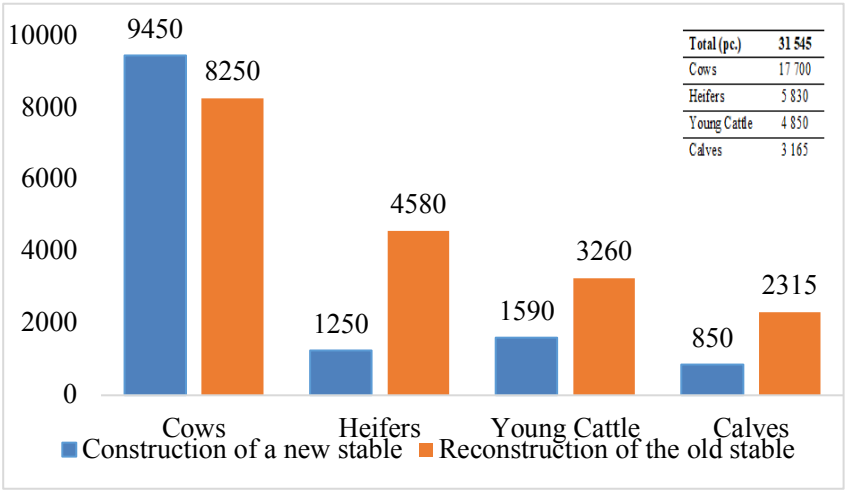

Figure 5 Planned capacity for different categories of bovine spieces. Note: Own processing based on questionnaire survey.

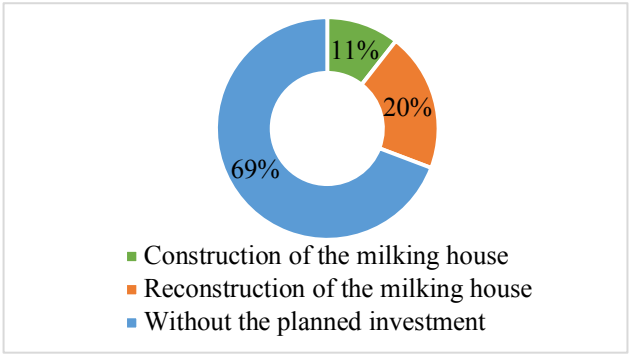

Figure 6 Objects for milking technology. Note: Own processing based on questionnaire survey.

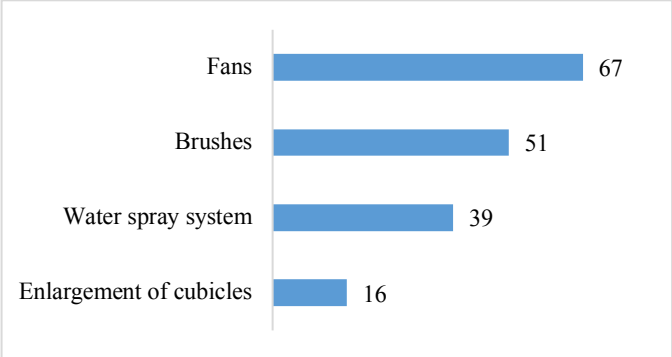

Figure 7 Planned welfare investments of primary milk producers in 2020. Note: Own processing based on questionnaire survey.

of housing investments in Slovakia is approx. 200 million €.

The investment in housing is really necessary for Slovakia because stables in which milk is produced are often in a bad condition. During our research, we found out that up to 77% of all stables were built between the years 1950 – 1990. These objects often do not fulfil the conditions for sufficient animal welfare, which may cause lower milk yields and thus affect milk deliveries.

Talking about Milking - objects for milking technology up to 69% of the respondents in 2020 do not plan investments, 20% of respondents plan to do some reconstruction and just 11% of respondents plan to build a new one (Figure 6).

Based on the survey, we found out that primary milk producers plan to invest in the purchase of milking robots (up to 10% of the respondents) in a total of 46 pieces in 2020. Milking robots are automatic milking systems where milking is performed without the direct participation of an operator **(Andrews, Davison and Pereira, 2016)**. It seems that milking robots are becoming popular systems also among Slovak farmers. This system relies on the use of the computers and the special herd management software, which means reducing labour cost from the partially manual milking.

However, according to **Shortall et al. (2016)** any decision to invest in automatic milking should consider several factors, such as the availability of skilled labour, lifestyle sought by the farmer, the interest in technology, and the initial capital investment requirement by the milking system.

The planned volume of investment for milking by our respondents is approx. 14 million €. Our estimated volume of objects for milking technology in Slovakia is approx. 35 million €.

Talking about investments in feeding and water feeding, we found out that most primary milk producers plan to invest in the fodder mixing wagon in 2020. On the contrary, the least interest is in investment into feeding robots. In recent years, great success has been made in the field of autonomous robotics. To date, autonomous robots have already been widely used in dairy farming that is a labour-intensive industry involving many tasks such as feeding, milking, livestock management, etc. Gradually, these robots appear to become huge advantages especially in aspects of increasing productivity, improving accuracy and decreasing labour costs **(Cheng, 2016).** In Slovakia, feeding robots are not popular among Slovak primary milk producers yet.

The planned volume of investment for feeding by our respondents is approx. 9 million €. Our estimated volume of feeding investments in Slovakia is approx. 25 million €.

In terms of the type of investments in technology and equipment to provide water for animals, our responders mainly utilize the water pumped from bored or dug wells or from free-flowing natural groundwater springs so their planned investment is primarily into water well drilling, reconstruction of hydro-globes (water towers), extension or construction of water distribution from an existing well or construction of a public water connection.

The planned volume of investment for water security by our respondents is approx. 1 million €. The estimated amount of investment for water security in Slovakia is 3 million €.

Regarding storage capacity, our analysed sample plans to invest in the reconstruction or construction of forage warehouses, feeding troughs, and hay-lofts. The estimated volume of investment of the analysed sample in the storage capacity is 9 million €. Our estimated amount of investment in the storage capacity in Slovakia is 20 million €.

Last but not least part of our research was animal welfare. In recent years, the animal welfare debate in livestock farming has continued to grow, including dairy cattle farming **(Koik, Thiele and Enneking, 2019)**.

According to **Buller and Morris (2003),** discussions about animal welfare generally begin with an often-unarticulated ethical assumption that it is morally acceptable for humans to use animals so long as they ensure that animals are free of physical and mental stress and able to experience positive feelings. On the other hand, animal rights movements consider any use of animals to be morally objectionable and argue for the development of dairy and meat alternatives **(Garner, 2016)**.

Understanding of animal welfare tends to include consideration of three aspects: (1) that welfare comprises animals' essential health and functioning (i.e. absence of disease and injury); (2) the need to consider animals' 'affective states' (such as pain, distress, and pleasure) and how positives and negatives add up to a quantitative indicator of well-being; and (3) animals' freedom to pursue 'natural' behaviours (e.g. grazing in the open air), including their ability to exercise control in a given situation to remove themselves from 'poor' situations and place themselves in more positive mental and physical states **(Fraser 2008; Ohl and Van der Staay 2012; Arnott, Ferris and O'Connell, 2017)**.

In literature about the welfare of animals, different authors have tended to emphasize different concerns. Given the complexity to understand the concept of animal welfare we understand it as a summary of three key points:

1) welfare comprises animals' essential health and functioning (i.e. absence of disease and injury);
2) the need to consider animals' 'affective states' – states like pain, distress, and pleasure as positive or negative add up to a quantitative indicator of well-being; and
3) the ability of animals to live reasonably natural lives by carrying out natural behaviour and having natural elements in their environment (e.g. grazing in the open air) **(Fraser 2008; Ohl and Van der Staay 2012)**.

An inadequate environment and breeding techniques mean that a significant proportion of livestock is in a state of chronic stress which greatly reduces the resilience, viability, longevity, production, and reproduction of genetically high-value animals. We must, therefore, respect the demands of animals to create the conditions for them to live and produce.

The number of days with extremely high temperatures in Slovakia is constantly increasing and according to the forecast, it is going to increase. Global warming significantly affects the animal's life which also affects how they are bred. Most breeders in Slovakia are not prepared for this. Therefore, they plan investment into modern technologies to reduce this negative impact of climate extremes. Appropriate ventilation is one such solution.

As you can see in Figure 7 primary milk producers plan to invest in different combinations of methods and practices of welfare in 2020. Respondents are most interested in fans. In

extreme summer conditions, when the air flow in the stables decreases and the air exchange is inadequate, breeders use fans to support the air flow. The second of planned welfare investment were brushes which improve the health of cows and have a very beneficial effect on blood circulation. At the same time, they increase the comfort and convenience of the cows, which affect milk production. Many responders also plan to invest in the water spray system to form an aerosol substance that cools the air. There were many answers with a combination of water spraying systems with fans which is a suitable solution to enhance the effect of air cooling. The responders are not certainly interested in the enlargement of cubicles (Figure 7).

The dairy sector is undergoing major structural changes in the EU. Current changes in the dairy sector affect farm efficiency, profitability, and long-term economic sustainability **(Cabrera, Solis, and Del Corral, 2010)**.

Dairy farmers need to invest to keep their farms in a good condition, maintain competitiveness in the market, increase the rate of technology adoption, and improve labour productivity **(Schick and Hartmann, 2005; Pouch and Trouvé, 2018)**.

In our research, we also identified the other forms of support to increase the competitiveness of Slovak milk producers such as:

1) Support investment in livestock production and new technologies of livestock production. Farm investment support is one of the EU Rural Development Programme policy instruments provided to enhance farm productivity, agricultural production efficiency and thus farm competitiveness **(Hurňáková, Bartová, and Fandel, 2016)**.
2) Create the same conditions for all EU milk producers. We believe that the new programming documents of the new CAP 2021 – 2027 will reflect this requirement. A new CAP delivery model should lead to a more results-based policy and greater flexibility in its implementation while preserving its common dimension **(Rossi, 2018; Matthews, 2018)**.
3) Linking science with practice and better cooperation with universities (educational and consulting activities, analyses, benchmarking). Many respondents would be interested in collaboration with universities. The biggest advantage of cooperation they see in the preparation of project documentation as well as in the analysis of various data.
4) Assistance in natural disasters. Natural conditions are one of the important factors affecting the economic competitiveness of dairy farming in relation to other agricultural activities **(Bórawski et al., 2020)**.
5) To raise awareness regarding the importance of milk production and milk consumption in the Slovak Republic. Milk production is important for society as a whole, both in terms of the economy of the state and the employment of the rural population. It is a traditional economic sector that due to its very favourable conditions promises a very viable future **(Gurčík, et al., 2016)**.

Finally, we would like to look at the last tested hypothesis, hypothesis 4. In **Error! Reference source not found.** below, we can see that the F-value is lower than the F-critical value for the alpha level selected (p = 0.05). Therefore, the null hypothesis is valid and sample means are equal, or they do not have any significant difference. Based on the One- way ANOVA we can say with a probability of 95% that the average milk yield per cow does not depend on the region.

Even though nonparametric methods show less power compared to parametric methods, we have decided to use the Kruskal-Wallis test for testing the last hypothesis. Similarly, the Kruskal-Wallis test did not confirm the existing differences between a region and an average milk yield per cow.

Within the individual regions of Slovakia, there is a different level in the development of the average milk yield per cow. Based on available data from previous years, Bratislava and Nitra regions significantly influence the achieved level of milk yield in Slovakia. On the contrary, in the east of Slovakia, the number of dairy cows is increasing every year.

On the member-farm level, the abolition of the EU milk quota system opened up new entrepreneurial options for farm growth. At times when milk prices were relatively high, such as in the years 2011 – 2014, many farmers invested in the expansion of their milk production.

Table 4 Anova:Single Factor.

Source of Variation	SS	df	MS	F	*p*-value	F crit
Between Groups	38035074.27	7	5433582	1.957673	0.068965	2.107506
Within Groups	263675400.6	95	2775531			
Total	301710474.8	102				

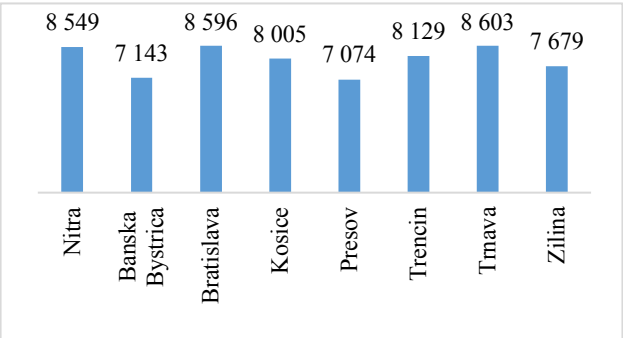

Figure 8 Anova: Single factor. Note: Own processing based on questionnaire survey.

However, when prices steeply declined in 2015 and 2016, the same farmers were not ready to accept the low prices offered to them by their cooperatives **(Nagy and Jámbor, 2019)**. The Slovak dairy market has been in an unfavourable situation in recent years. Long-term low prices of raw cow milk led to the liquidation of primary milk producers **(Váryová et al., 2019)**. The year 2019 was one of the better years for primary milk producers. After some time, the situation has improved, mainly thanks to the recovery of milk purchasing prices (32.6 cents per kilogram over the year) but also thanks to some support **(Štefániková, 2020)**.

CONCLUSION

Although the situation in the milk sector is improving, not all primary milk producers achieve the balanced cost of milk production. No one is producing milk at the same cost. Several factors affect milk production. It is different to produce milk in mountainous areas and the flat south, so natural conditions also affect it.

In our research, we pointed out the current situation and needs of milk producers in Slovakia in relation to the strategic objective – to increase the competitiveness of the milk producers. The sample we examined plans to increase the number of milk deliveries by 2.6% in 2020. This fact is very positive for the sector. On the other hand, costs have risen again, either because of rising energy prices or because of rising labour costs caused by the government measures and the conditions in which the milk is produced are insufficient. The oldest stables in which dairy cattle are kept exist on average 50 years. We certainly need to make our primary milk producers more competitive. In the upcoming period, we need a government that will prioritise and support the development of the dairy sector.

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CONSUMER PREFERENCES AND DECISION-MAKING DETERMINANTS FOR THE PURCHASE OF SHEEP'S MILK AND ITS PRODUCTS

Ľudmila Nagyová, Andrej Géci, Elena Horská

ABSTRACT

When observing consumer behaviour, we find that the consumer carries out the process of purchasing decisions under the influence of several factors. These factors come from the external environment, from its individual characteristics, and also from the specific purchasing situation. The above facts show that consumer preferences and understanding of the behaviour is a very demanding process. This is mainly because consumers may behave differently, i.e. they may have different consumer behaviours that depend on their needs and desires. Therefore, it is necessary to know the factors that influence consumer behavior or the purchasing decision for certain market commodities. The presented research document is focused on the issue of consumer behavior and decision-making in sheep's milk and its products. Sheep's milk has a long tradition and is one of the basic building elements of human food. Consumer behaviour is constantly evolving, their needs and desires are changing as well as factors that influence their shopping behaviour. The main goal of the research is to draw attention to the personality of the consumer, to selected personality characteristics and social factors, and, subsequently, to evaluate their impact on purchasing behaviour and making purchasing decisions on the market of sheep's milk products. Primary data were obtained from a survey conducted on a sample of 796 respondents. Most of the respondents were classified in the sanguine group based on temperament - an emotionally stable extrovert. When buying sheep's milk and its products, they make emotional decisions (57.17%). The most important factor in buying these products was their quality (47.16%). Assumptions were formulated for deeper analysis, which was subsequently verified by the means of a statistical test - Chi-square of the square contingency. The degree of dependence between the examined variables was determined by the means of the Cramer contingency coefficient.

Keywords: consumer; sheep´s milk; sheep´s milk products; consumer behaviour; decision-making determinants

INTRODUCTION

Humans develop during their lives. They learn new things, come up with new ideas and thoughts, and ultimately change their behaviour **(Christensen et al., 2014)**, behaviour towards other people in their lives but also their behaviour when buying common goods of daily consumption. On the one hand, shopping methods, preferences, tastes are changing, and on the other hand, the determinants of the influence on their shopping behaviour are constantly changing **(Yadav and Pathak, 2017)**. In general, consumers develop over their lifetime. Currently, most consumers are becoming rational and conscious, as they live in a very fast period **(Vuelvas and Ruiz, 2017)**. Recent years have shown that consumers pay close attention to what they put in their shopping cart when shopping **(Ertemel and Ammoura, 2019)**. The authors **Rana and Paul (2017)** claim that in recent years there has been a growing consumer demand for healthy and especially high-quality food. This interest is causing a positive year-on-year growth in the market for organic products.

Consumer, their behaviour and decision-making

A consumer is every person whose primary task is to satisfy needs and desires through the purchase of various goods and services **(Javornik, 2016)**. These needs and desires can be his/hers but also another family member. In this case, when a family member buys goods and consumes them at the same time, we are talking about the consumer **(Biswas and Roy, 2015)**. According to **Lemon and Verhoef (2016)**, there is another type of consumer and that is the customer. For them, only a shopping act is typical. The customer does not get to the consumption and satisfaction of needs through purchased goods **(Dabholkar, 2014)**. On the contrary, a person who only consumes pre-purchased goods and products without purchasing activity is called a consumer **(Goodman and Paolacci, 2017)**. Each person has gone through all phases

of purchase and consumption in their lives. Based on the above, consumer behavior is a constantly changing process, the basis of which is the reflection of external but mainly internal attributes **(Trudel, 2018)**. Consumer behaviour is defined by **Drossos et al. (2019)** as the intentional conduct of individual purchasers, whose primary task is to satisfy their needs. They try to meet these needs at the highest possible level **(Coronel et al., 2019)**.

Determinants of consumer behaviour

Various factors play a role in enabling consumers to make the right purchasing decisions. These factors often operate at the unconscious consumer level **(Victor et al., 2018)**. One of the main influences on the consumer when buying is still the price of goods or services. The monetary expression of the value of goods is perceived most among consumers whose incomes do not reach high numbers **(Malc et al., 2016)**. According to **Roux et al. (2017)**, however, solvent customers are much less prone to the pricing value of goods and services. Another important determinant is the product brand. Consumers are fond of reaching for familiar and already tried and tested products. If they are satisfied with a particular good or service, it is very difficult to get their affection to focus their attention on a competing product and to buy and try it **(Godey et al., 2016)**. In the research by **Madzharov et al. (2015)**, who discussed the relationship between the fragrance and space, the increased interest in premium and branded products was confirmed. The authors state that the smell influences the spatial perception of consumers in the shopping environment, which affects the feeling of the power of customers and thus the preferences of individual products and, ultimately, their shopping behaviour. Impulsive behaviour is also a very important determinant. **Holly (2019)** states that while this form of shopping behaviour will bring greater satisfaction to the seller, in the end, consumer behaviour is satisfied at a lower level. Very important determinants of the impact on consumer behaviour are consumer preferences, food trends, and also eating habits. In recent years, as a result of the growing interest in health, there is a preference for quality and organic products **(D´Amico et al., 2016)**. Last but not least, the basic determinants include sufficient information that determines the purchase. **Jiang and Yang (2018)** argue that when looking for basic information about the products, the consumer's purchase is influenced by their needs, values, and interests. Therefore, individual companies need to build a certain competitive advantage. To achieve such an advantage, consumers must receive food products of different quality and the necessary information, which would allow consumers to make their choice easier **(Gracia and Magistris, 2016)**. An important step in the decision-making process is also the attention that is a prerequisite for the consumer to thoroughly process the information **(Drexler et al., 2017)**. Consumers also have the opportunity to use heuristics (methods of problem-solving) to simplify their decisions, through which they do not pay the required attention to all product features **(Loo et al., 2015)**. Based on the above factors, it is interesting to analyze consumer determinants that lead to justification and understanding of consumer behaviour **(Aquilani et al., 2015)**.

Sheep's milk and its products

The present document also deals with the consumption of sheep's milk, in particular with the cheeses made from this type of milk. In general, we can encounter various reasons why people do not consume this milk **(Kurajdová et al., 2015)**. The most important reason is the content of lactose in milk and its products. The lactose content is higher in cow's milk products, as it decomposes in individual product processing processes. This process does not take place in sheep's milk products and therefore the milk and its products are also intended for people suffering from lactose intolerance **(Balthazar et al., 2017)**. Concerning the content of fat and vitamins, the fat of sheep's milk is twice as high and the content of vitamins is higher than that of cow's milk **(Revilla et al., 2017)**. A negative attitude towards the consumption of sheep's milk products may also stem from one's own beliefs but also the lifestyle of consumers **(Griebler et al., 2016)**. The development of sheep breeding in Slovakia is constantly changing. Since 2013, it has been constantly declining. In 2019, the number was at the level of more than 351 thousand head of sheep. Compared to the previous year, this is a decrease of more than 14,000 head **(Horalová, 2019)**. Based on the decline in sheep farming, it is clear that sheep milk production is also declining. According to **Horalová (2018)**, consumption in 2018 was at the level of 1.3 million kg. As for world sheep milk production, it is headed by Greece, followed by Spain, Italy, and Romania **(Palo, 2015)**. According to the **Ministry of Agriculture and Rural Development of the Slovak Republic (2019)**, the estimated consumption of sheep's milk per capita is on average around 2 kg (in 2018 it was 1.96 kg per capita). The average selling prices of sheep's milk according to **Huba et al. (2018)** for the year 2018 are around 1 Euro (specifically 0.98 EUR per liter of sheep's milk).

Scientific hypotheses

Assumption n. 1: We assume that there is a relationship between the age of the respondent and the consumption of sheep's milk.

Assumption n. 2: We assume that there is no relationship between the respondent's temperament and the factor that most influences them when buying sheep's milk.

Assumption n. 3: We assume that the personality in terms of emotional stability/lability has an impact on the emotional behaviour and decisions of consumers on the food market.

Assumption n. 4: We assume that the consumer's extroverted/introverted personality does not affect their emotionality in food market behaviour and decisions.

Assumption n. 5: We assume that the type of consumer temperament influences their emotional behaviour and decision-making in the food market.

MATERIAL AND METHODOLOGY

Based on the findings of consumer behaviour on the market of sheep's milk and its products, a questionnaire method was used. The survey was conducted online using the Google Forms tool. Data acquisition was carried out at the turn of two years (2019 – 2020) and took place in the Slovak Republic. The sample was 796 respondents. The survey was divided into four parts. The first part focused

on the Eyesenck personality test, which determines the temperament, emotional lability/stability as well as extroversion/introversion. The second part focused on factual issues related to consumer behaviour when buying food. This part focused on consumer behavior on the market, i.e. whether they act emotionally or rationally. The third part focused on specific-factual issues, which were focused on the consumption of sheep's milk, consumer preferences, and also on the factors influencing the purchase. The last part of the questionnaire survey was focused on classification questions. In the beginning, it was necessary to process and evaluate Eyesenck's personality questionnaire. To evaluate the test, a publicly available and in practice used evaluation method was used - tabular application of values in a template, which is based on the horizontal and vertical axes.

Table 1 Rating scale intervals – temperament.

Type of temperament	Rating scale on the Y-axis	Rating scale on the X-axis
Melancholic	<13 – 24>	<0 – 11>
Choleric	<13 – 24>	<13 – 24>
Phlegmatic	<0 – 11>	<0 – 11>
Sanguine	<0 – 11>	<13 – 24>
Uncertain	= 12	= 12

Table 2 Rating scale intervals – emotionality.

Consumer	Rating scale
Rational	<1 – 24>
Emotional	<24 – 40>

Table 3 Characteristics of Respondents.

Category of Respondents	%	Place of Residence	%
Male	34.30	City	51.88
Female	65.70	Village	48.12
Age Structure	**%**	**Net monthly income (€)**	**%**
Up to 26 years	41.33	Up to 500	41.08
27 – 33 years	16.33	501 – 800	28.77
34 – 45 years	18.84	801 – 1 000	14.20
46 – 55 years	14.07	1 001 – 1 500	10.43
56 – 62 years	4.77	1 501 and more	5.53
63 and more years	4.65		
Education	**%**	**Economic activity**	**%**
Primary school	4.40	Student	26.88
Vocational school	6.91	Employed	59.92
Secondary school	43.47	Unemployed	6.66
University	45.23	Retired	6.53

The position of the resulting point (Table 1) represents the location in one of the four quarters, with each quarter representing a different temperament.

This was followed by a survey of consumer behaviour to assess consumers' emotional reactions, views, and attitudes in the sheep's milk market. The finding is based on a scale as an attitude test. It includes 8 items for which the respondents indicate the degree of their agreement or disagreement with the above statements concerning behaviour and decision-making when purchasing food products. A Likert 5 point scale was used (1 – strongly disagree, 2 – disagree, 3 – don't know, 4 – agree, 5 – strongly agree). Within this assessment, a gross emotionality score has been calculated, which can be in the range of 1 to 40. The higher the score, the higher the tendency of the consumer to behave emotionally. Individual respondents were evaluated based on a specified interval of the rating scale (Table 2).

The structure of the obtained data according to the gender was as follows - 65.70% of women and 34.30% of men. The age structure was divided into 6 age categories. The age structure up to 26 years had the largest share (41.33%). The highest achieved education was at the university level (45.23%). Regarding the economic status of the respondents, most of them (59.92%) are employed. Within the net monthly income per respondent, the largest interval turned out to be an income not higher than EUR 500 (41.08%). Most of the respondents stated the city as their residence and most respondents live in the Nitra region. More information on the structure of respondents is given in Table 3.

Statistic analysis

At the beginning of the questionnaire survey, the scientific assumptions were set, the validity of which would be verified using selected statistical methods. Contingency tables were applied to the obtained primary data and subsequently, the data were evaluated by the means of qualitative statistics - the Chi-square test of good agreement. The degree of dependence between the examined variables was determined by the means of the Cramer contingency coefficient.

If the calculated test criterion $\chi 2$ is higher than or equal to $\chi 2$ for the significance level $\alpha = 0.05$ and degrees of freedom (r-1) * (s-1), then the H_0 hypothesis is rejected, which means that there is a relationship between the given variables. Conversely, if the calculated test criterion $\chi 2$ is less than $\chi 2$ for the significance level $\alpha = 0.05$ and degrees of freedom (r-1) * (s-1), then H_0 hypothesis is accepted, which means that there is no dependence between the given variables.

RESULTS AND DISCUSSION

The research was implemented to identify the temperament, extroversion/introversion of the respondent or emotional stability/lability, and then define their impact on emotional or rational behaviour on the sheep's milk market and also to ascertain consumer attitudes towards the sold milk products. Therefore, it was necessary to categorize respondents at the beginning of the research. The sorting was carried out based on the question of whether they consume sheep's milk and sheep's milk

products. Thus, the respondents who answered positively to the above question continued. 642 respondents (80.65%) answered this question in the affirmative. The first part of the research was focused on determining the temperament, extroversion/introversion, and also on emotional stability/lability through Eyesenck's personality questionnaire. Figure 1 shows approximately the same proportion of unstable and stable individuals in the sample of respondents. Emotionally stable people predominate (345 respondents), which is 54.33%. On the contrary, in a smaller proportion (297 respondents), the respondents are emotionally unstable.

Based on the above-obtained results, we can state that a large number of people do not have neurotic tendencies, i.e. they are emotionally stable. They are characterized by less sensitivity and suspectivity to other people. They are characterized by endurance and resistance, they react calmly and are not subject to impulsive reactions. They consider decisions precisely and have their emotions and feelings under control. On the other hand, there are people with neurotic tendencies, which represent their lability. They are more sensitive and easier to be swayed. Almost identical results were found in a research document by **Rybanská (2015)**. The author discusses the fact that both groups of people are important for marketing research, as it is necessary to properly set up and target a marketing strategy along with communication to a selected segment of consumers.

The statistical observation was made on an assumption whether the personality in terms of emotional stability / lability has an impact on the emotional behavior and decision-making of the consumer on the food market.

H_0: We assume that personality in terms of emotional stability/lability does not influence the emotional behavior and decisions of consumers in the food market.

H_1: We assume that personality in terms of emotional stability/lability has an impact on the emotional behavior and decisions of consumers in the food market.

We have used the Chi-square test of square contingency to verify the hypotheses. In this case, the tabular value was at the level of 3.84 and the test characteristic was calculated at the level of 12.20, which means that we reject the null hypothesis. We accept the alternative hypothesis and claim that the personality in terms of emotional stability / lability influences the emotional behavior and decisions of consumers on the food market. Based on the test results, we consider the assumption to be correct. The strength of the observed dependence was subsequently investigated with the use of the Cramer correlation coefficient (0.17), based on which it can be stated that there is only a small correlation / weak dependence between the analyzed variables.

Figure 2 shows approximately the same proportion of introverted and extroverted respondents. Out of the total number of filtered respondents, we refer to 347 respondents as extroverts. The remaining more than 45% of respondents can be ranked based on a personality test among introverts (295 respondents).

Based on the above results, we can conclude that a larger number of respondents are extroverts. Impulsive action is typical for this type of personality (they act first and then

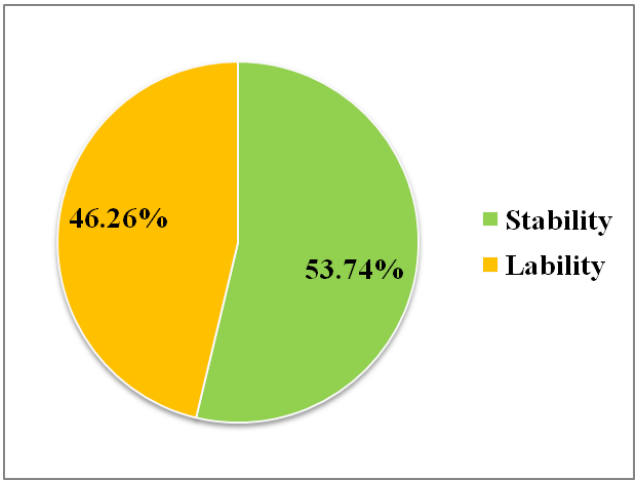

Figure 1 Structure of respondents according to the scale of extraversion.

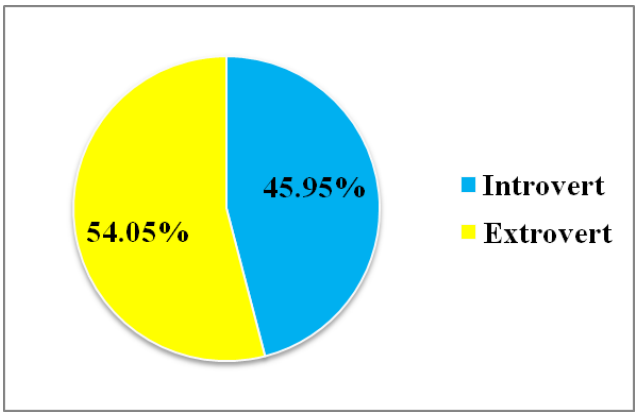

Figure 2 Structure of respondents according to the scale of extraversion.

think), they have a strong need for contact with the external environment. They also like diversity, they are open and motivated towards other people and things. On the contrary, the introverts are motivated internally, they are trying to establish an authentic connection with the others. The team of authors **Lu et al. (2015)** concluded that even with this division of people, one mentioned group of people in marketing research cannot be forgotten, as their response to stimuli is different.

A statistical observation was made on the established assumption that the extroverted / introverted personality of the consumer does not influence his emotionality in behaviour and decision-making on the food market.

H_0: We assume that the consumer's extroverted/introverted personality does not affect his/her emotionality on food market behaviour and decision-making.

H_1: We assume that the consumer's extroverted/introverted personality influences his/her emotionality on food market behaviour and decision-making.

To verify the hypotheses, we have used the Chi-square test of square contingency. In this case, the tabular value was equal to 3.84 and the test characteristic was calculated at 0.18, which means that we reject the alternative hypothesis. We accept the null hypothesis and claim that

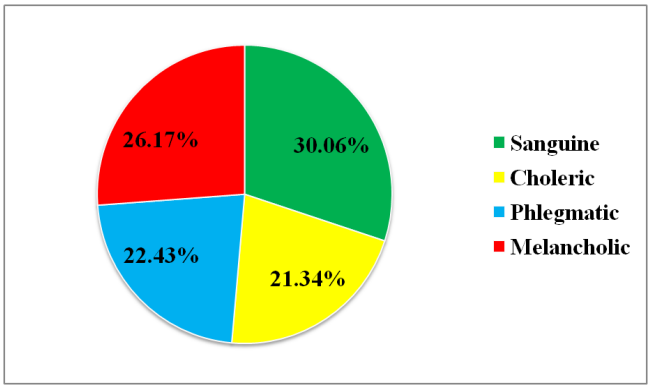

Figure 3 Structure of respondents according to temperament.

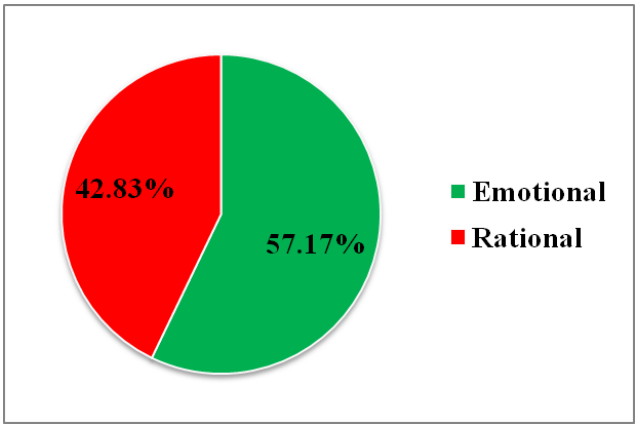

Figure 4 Emotional vs. rational consumer behavior.

the consumer's extroverted/introverted personality does not affect his/her emotionality on food market behavior and decision-making. Based on the test results, we consider the assumption to be correct.

If we reconcile the above dimensions of the personality test (emotional stability/lability and extroversion / introversion), Hippocrates' 4 types of temperament (phlegmatic, sanguine, choleric, and melancholic) can be confirmed. The distribution according to temperament is shown in Figure 3.

The most represented group among the evaluated respondents are sanguine, who is represented by 193 respondents in the total number. The team of authors **Ul Islam et al. (2017)** claims that they are characterized by high mental activity and performance, but also by a rapid appearance and alternation of emotions. The second most represented type of temperament is melancholic (168 respondents). This type is characterized by a slow course of emotions, but with great intensity and longer duration. The third group of temperament was a phlegmatic (144 respondents). Low levels of mental activity are typical for phlegmatic. The last group of temperaments is choleric people (137 respondents), who are characterized by a high level of mental activity, energetic action, sharpness, short temper, the strength of movements, fast, pace and irascibility.

The second part of the survey was focused on identifying the emotionality manifested in the consumer behaviour of respondents. The task was to classify the respondents into two groups, namely emotional and rational consumers (Figure 4). In this section, 8 statements were presented, in which the respondents had to mark their answers in the form of a Likert scale (assign points from 1 to 5).

Figure 4 shows that emotional consumers (367 respondents) are more represented in the research sample. They are characterized by the fact that they are strongly influenced by emotions when making purchasing decisions, their rational choices remain slightly in the background. The second group is rational consumers (275 respondents). Emotions do not have a significant effect on this type of person The team of authors **Dwivedi et al. (2019)** states in their research that the fact of how they feel when buying remains in the background. It is typical of them that they consistently consider all possible alternatives and carefully compare them.

A statistical observation was made on the established assumption - that the type of consumer temperament influences his/her emotional behaviour and decision-making on the food market.

H_0: We assume that the type of consumer's temperament does not influence his/her emotional behaviour and decision-making in the food market.

H_1: We assume that the type of consumer's temperament influences his/her emotional behaviour and decision-making in the food market.

To verify the hypotheses, we use the Chi-square test of square contingency. In this case, the tabular value was 3.84 and the test characteristic was calculated at 18.30, which means that we reject the null hypothesis. We accept the alternative hypothesis and claim that the type of consumer temperament influences his/her emotional behavior and decision-making in the food market. Based on the test results, we consider the assumption to be correct. The strength of the detected dependence was subsequently investigated with the use of the Cramer's correlation coefficient (0.18), based on which it can be stated that there is only a small correlation or weak dependence between the analyzed variables.

The last part of the research was focused on consumer behaviour on the market of sheep's milk and its products. In this part, we investigated consumer behaviour to the purchase of specific types of sheep products, reasons for consumption, the places of purchase, factors influencing the purchase, and also factors that would increase the consumption of these specific types of food. The first question in this part was focused on the specific types of sheep 's milk products purchased (Figure 5).

It follows from the above figure that most respondents prefer sheep cheese (197 respondents), other popular and consumed products are parenica (steamed cheese), nite (string cheese), and korbáčiky (braided string cheese), which were mentioned by 164 respondents (12.81%). Oštiepok (cheese preserved by smoked cure) was purchased by 138 respondents, i.e. 17.34% of the total number of respondents. This was followed by hard and lump cheeses – 164 respondents (20.60%), sour milk (sheep´s whey) – 69 respondents (8.67%) and yogurts (76 respondents). The least interest among consumers is in sheep's milk, which is bought by only 6.28% of respondents (50 respondents).

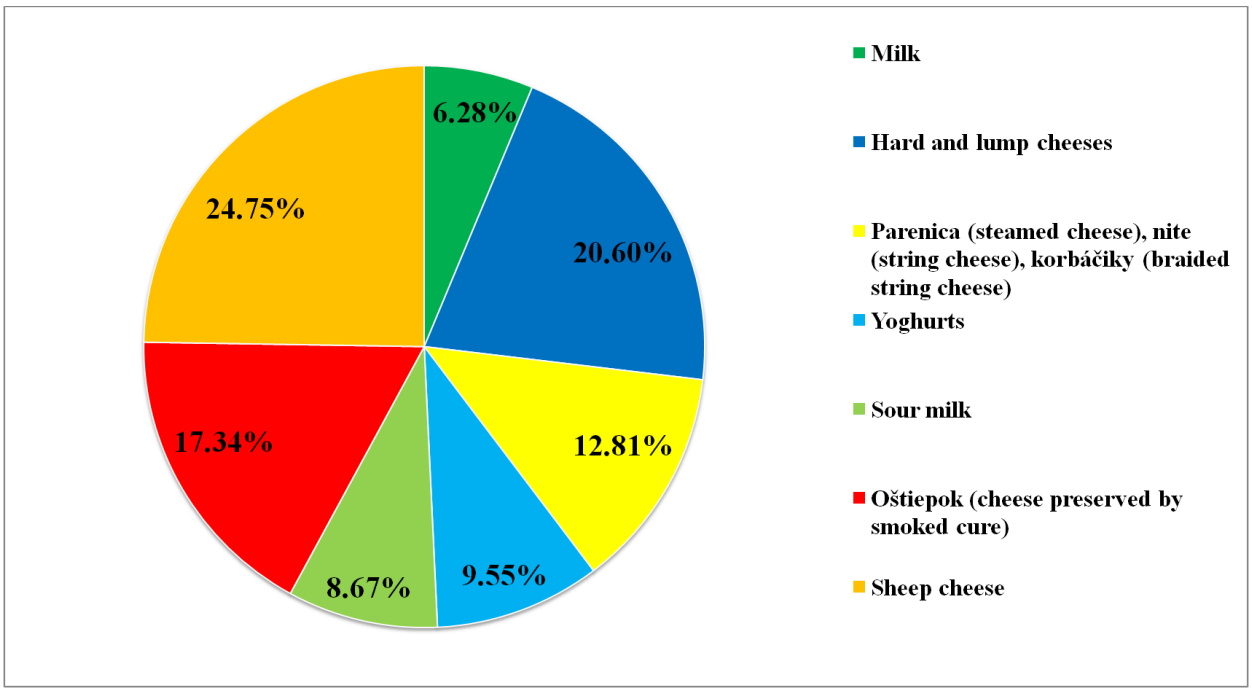

Figure 5 Purchased types of sheep products.

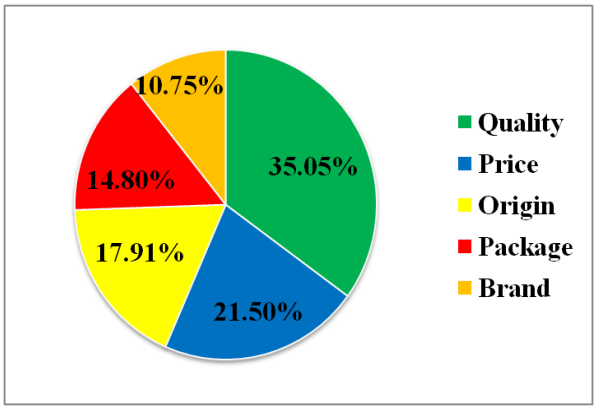

Figure 6 Factors influencing the consumer when buying sheep products.

A statistical observation was made on the established assumption - whether there is a relationship between the age of the respondent and the consumption of sheep's milk.

H_0: We assume that there is no relationship between the age of the respondent and the consumption of sheep's milk.
H_1: We assume that there is a relationship between the age of the respondent and the consumption of sheep's milk.

To verify the hypotheses, we use the Chi-square test of square contingency. In this case, the tabular value was 11.07 and the test characteristic was calculated at 39.40, which means that we reject the null hypothesis. We accept the alternative hypothesis and claim that there is a relationship between the age of the respondent and the consumption of sheep's milk. Based on the test results, we consider the assumption to be correct. The strength of the observed dependence was then investigated using the Cramer correlation coefficient (0.34), based on which it can be stated that there is a small almost medium correlation / dependence between the analyzed variables.

Another issue dealt with the reasons for consuming sheep's milk and sheep's milk products. Most respondents answered that the reason for consuming these products is their taste (55.76%). This was followed by the health factor (23.21%), nutritional value (19.47%), and finally other reasons for consumption, which were identified by 10 respondents (most often they reported consumption based on support for sheep breeding in Slovakia).

This was followed by a question that focused on the specific purchase of sheep's milk and its products. We, therefore, asked where specifically (which store) consumers buy these products. They most often make purchases in retail chains (48.44%). The main advantage of retail chains is time availability, which is a very important criterion for more and more buyers. Discounts and supermarkets, as a modern type of store in the vicinity, are gaining in importance, and buyers shop here more often. On the contrary, the importance of small and counter stores is declining and specialized stores are defending their position only with difficulty **(Silver et al., 2016)**. Another shopping place is local stores (29.13%) and the last place was a specialized store (22.43%).
The penultimate question of inquiry followed, and these were the factors that influence the consumer when buying sheep products (Figure 6).

The most significant influence on the purchase of sheep products is quality (225 respondents). Then the price (138 respondents), origin, and packaging follow. In the last place was the brand factor, which was marked by 69 respondents.

A statistical observation was made on the established assumption – whether there is some dependence between the respondent's temperament and the factor that most influences them when buying sheep's milk.

H_0: We assume that there is no relationship between the respondent's temperament and the factor that most influences them when buying sheep's milk.

H_1: We assume that there is a relationship between the respondent's temperament and the factor that most influences them when buying sheep's milk.

To verify the hypotheses, we use the Chi-square test of square contingency. In this case, the tabular value was at the level of 21.03 and the test characteristic was calculated at the level of 11.54, which means that we reject the alternative hypothesis. We accept the null hypothesis and claim that there is no relationship between the respondent's temperament and the factor that most influences them when buying sheep's milk. Based on the test results, we consider the assumption to be correct.

Most respondents know that the consumption of sheep's milk and sheep's dairy products is low in Slovakia. Therefore, the last question was focused on the reasons that would lead to higher consumption of sheep's milk and its products. Most respondents stated that the reason that would increase their consumption is the greater availability of products (25.86%). The second factor was a lower price (24.61%), followed by better promotion (22.43%) and higher quality (17.91%). Other respondents indicated a different option saying they would not be motivated by anything.

CONCLUSION

The survey showed that more than half of the respondents are emotionally stable (53.74%). Within the personality type, there is a higher proportion of extroverts (54.05%). The sanguine was most represented within the temperament (30.06%) and emotional types of respondents predominate (57.17%) within decision-making based on emotions.

In 2016, the Commodity Council for Sheep and Goats of the Ministry of Regional Development of the Slovak Republic stated that consumer interest in sheep products is increasing. Based on the results, it can be stated that the consumption of sheep's milk and sheep's products is not so high in Slovakia. More than 80% of respondents in the study consume sheep's milk and sheep's milk products. The rest of the respondents do not consume these products and the most common reasons were unpopularity, preference for cow's milk over sheep´s milk, health reasons such as the allergy or intolerance, as well as high prices or unavailability. The most frequently consumed and also purchased sheep product is sheep cheese (24.75%), which is considered a traditional Slovak product. Other popular and consumed products among people are cheeses (20.60%), oštiepok (cheese preserved by smoked cure) – (17.34%). The research also found that consumers in the market of sheep's milk products use mainly retail chains as a place of purchase (48.44%). According to the respondents, the most important factor that affects the purchase of these products was their quality (35.05%). According to consumers of the survey, the price also has a significant impact (21.50%). An important finding was to obtain information about what would motivate consumers or would lead to higher consumption of sheep's milk and its products. The research shows that most respondents would welcome more accessibility (25.86%), followed by a lower price (24.61%) and better promotion (22.43%).

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FUNCTIONAL PROPERTIES OF THREE NATIVE STARCHES AND THEIR MODIFIED DERIVATIVES

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ABSTRACT

Starches were isolated from cocoyam (*Xanthosoma sagittifollium*), white yam (*Dioscorea rotundata*) and bitter yam (*Dioscorea dumentorum*). Starch modification was carried out using acetic anhydride and phthalic anhydride. The native and modified starches were characterized using Fourier Transformed Infra-red Spectroscopy (FTIR) for identification of the functional groups. Functional properties such as water absorption capacities, oil absorption capacity, swelling power, solubility, gelation temperature, least gelation capacity, amylose content and pH were determined using standard procedures. Acetylation increased the water absorption capacity, oil absorption capacity, swelling power, amylose content, and solubility of the starches while phthalation decreased water absorption capacity, oil absorption capacity, swelling power, and solubility of the starches. Native cocoyam starch has the highest gelation temperature (85 °C) while Acetylated bitter yam has the lowest gelation temperature (74 °C). The pH of the native and modified starches was within the range of 4.14 − 6.55. Phthalation and acetylation increased the bulk density of the starches. Native cocoyam, white yam, and bitter yam starches had the lowest gelation concentration (6%). Modification of native starches will improve the usage of starch in food and non-food applications.

Keywords: native starch; phthalation; gelation; amylose; pregelatinized starch

INTRODUCTION

Starch is one of the most available natural polymers is a starting material or an intermediate for many chemical industries **(Yadav and Garg, 2013)**. Starch is a useful raw material for adhesive industries because of its availability and abundance **(Yu et al., 2009)**. Starch has been applied as a filler and bonding agent in the making of tablets, it is also used as an additive to improve the shelf life of soaps and detergents. Other uses of starch are in the rubber and foam industries **(Tonukari, 2004)** and the food industry **(Dura and Rosell, 2016)**.

In the food sector, starch is being used to give divers functionalities which include stabilizing, gelling, encapsulating, thickening, texturing, and shelf-life elongation. Despite the numerous advantageous properties in chemical industries, however, many starches in their crude form have limited applications in industrial processes as they have a high level of retrogradation which limits their application in food processing industries **(Singh, Kaur and Singh, 2004)**. However, it is necessary to modify crude starches to incorporate some specific properties which thereafter make them useful in the industrial sector **(Torruco-Uco and Betancur-Ancona, 2007)**.

Cocoyam (*Colocasia esculenta*) which is an ancient tuber of *Araceae* family originated from South-East, Asia, and has been cultivated for over 2000 years **(Wang, Truong and Wang, 2003)**. It has both red and white varieties. White yam (*Dioscorea rotundata*) is widely cultivated in Africa as an edible tuber with economic importance **(Omonigho and Ikenebomeh, 2000)**. Bitter yam (*Dioscorea dumetorum*) belongs to *Dioscoreacae* family. It is rich in phyto-nutrients such as proteins **(Medoua et al., 2005)**; still, it remains one of the underutilized tropical tubers in the world **(Owuamanam et al., 2013)**.

The present study focuses on the modification of four different starches using acetylation and phthalation processes, and also the determination of the functional properties of both native and the modified starches.

Scientific hypothesis

This research evaluated the significance of chemical modifications of native starches and the subsequent effects by comparing the characterizations and properties with their modified derivatives.

MATERIAL AND METHODOLOGY

Materials

Cocoyam, white yam, and bitter yam (Figure 1) were bought from a local market in Ado-Ekiti, Ekiti State, Nigeria. All the reagents used were of analytical grade.

Figure 1 Pictorial representations of cocoyam (A), white yam (B) and bitter yam (C).

Starch Isolation: The starches were isolated using the wet extraction method as described by **(Shujun et al., 2006)**. The tubers were thoroughly washed with water, cut into small sizes, and homogenized blended for about five minutes. The produced slurry was transferred into a muslin cloth and released into a bucket with distilled water. The content was continuously squeezed to eject the starch into the bucket of water, the starch was allowed to settle overnight and the supernatant was decanted. The product was rinsed continuously to remove soluble impurities until the supernatant was clear, the final product was spread on a flat substrate and air-dried.

Preparation of acetylated starches

The method of **(Sathe and Salunkhe, 1981)** was employed in the acetylation process. 100 g of starch was measured and dispersed in 500 mL distilled water, the resulting mixture was magnetically stirred for 20 min. The pH of the obtained slurry was adjusted to 8.0 using 1 M NaOH. 10.2 g of acetic anhydride was added for a period of 1 h, and the reaction was allowed to proceed for 5 min after the addition of acetic anhydride. The pH of the slurry was then adjusted to 4.5 using 0.5 M HCl. The product was filtered, washed several times with distilled water, and air-dried.

Preparation of Pregelatinized Starch Phthalate

Pre-gelatinized Starch Phthalate was prepared using the method of **(Surini, Ssputri and Anwar, 2014)**. Two basic steps were involved: gelatinization and esterification. Gelatinization was carried out by heating starch solution at 70 °C, the gel was then oven-dried, ground, and sieved. The esterification reaction was done by reacting 10% pre-gelatinized starch in distilled water with 16.7% solution phthalic anhydride in 96% ethanol. 10 M NaOH was added continuously during the reaction to keep the pH between 8 and 10. Anhydrous sodium sulphate was added to absorb excess moisture. Stirring was carried out at 1000 rpm, the stirring was continued for 30 more minutes and allowed to stay for 24 h. The mixture pH was adjusted to 6.5 − 7.0 using HCl solution. 50% Ethanol was added into the neutralized solution to wash the un-reacted phthalate. The final precipitate was dried, ground, and sieved to obtain pre-gelatinized starch phthalate (PCSP) powder.

Fourier Transform Infrared (FT-IR) Analysis

The functional groups of native and modified cocoyam, bitter yam, and white yam starches were obtained using Fourier Transform Infra-Red (Shimadzu Model FTIR – 8201PC).

FUNCTIONAL PROPERTIES

Water absorption capacity (WAC)

Water absorption capacity was carried out using the method described by **(Omojola et al., 2010)**. One gram of the sample was mixed with 10 mL distilled water for 5 min. The sample was allowed to stay for 30 min, centrifuged at 3000 rpm for 30 min, the volume of the supernatant was measured. Assuming the density of distilled water was 1 $g.mL^{-1}$.

Swelling power and solubility

Swelling power and solubility were determined using the method described by **(Awokoya et al., 2011)**. One gram of native starch was weighed and transferred into a clean and dried test tube (W1). The native starch was dispersed in distilled water (20 mL). The obtained slurry was heated at 60 ºC for 30 min in a calibrated water bath. The mixture was centrifuged at 3000 rpm for 20 min, the supernatant was decanted and the swollen granules were weighed

(W2). 10 mL of the supernatant was oven-dried at 120 °C. The residue obtained on drying the supernatant indicates the quantity of starch solubilized. The swelling and solubility are calculated as follows:

$$Swelling\ of\ starch\ (g/g) = \frac{W_2 - W_1}{weight\ of\ starch}$$

$$Solubility\ of\ Starch\ (g/g) = \frac{weight\ of\ dried\ aliquot}{weight\ of\ starch}$$

Gelatinization temperature (GT)

Gelatinization temperature was determined by the method described by **(Attama et al., 2003)**. About 1 g of starch sample was transferred into a beaker and 10 mL of distilled water was added. The dispersion was heated on a hot plate. The gelatinization temperature was then taken with a thermometer suspended in the slurry.

Determination of least gelation concentration

The method of **(Sathe and Solunkhe, 1981)** was used with slight modification. Appropriate sample suspensions of 2, 4, 6, 8, 10, 12, 14, 16 w.v^{-1} were prepared in 5 mL distilled water. The test tubes containing the suspensions were heated for 1 h in boiling water cooled under running tap water. The least gelation concentration was determined as concentration when the sample from the inverted test tube did not fall down or slip.

Determination of bulk density

Bulk density was determined using the procedure of **(Gbadamosi and Oladeji, 2013)** with slight modification. The sample (10 g) was put into a 100 mL graduated cylinder. The cylinder was tapped forty times and the bulk density was calculated as weight per unit volume (g.mL^{-1})

$$Bulk\ density\ (g/mL)\frac{W_2 - W_1)}{V}$$

pH Determination

The pH was determined using the procedure of **(Omojola et al., 2012)**. 20 g of the sample was shaken in 100 mL of distilled water for 5 min and the pH was determined using a pH meter.

Amylose content

The amylose content of the samples was determined by the colorimetric measurement of the blue amylose-iodine complex **(Mir, Srikaeo and García, 2013)**. In summary, 100 mg of sample was weighed into a 100 mL volumetric flask, mixed with 1 mL ethanol and 9 mL of 2 M NaOH. The samples were diluted and the iodine solution was added. After 10 min incubation at room temperature, the absorbance at 620 nm was analyzed using UV-spectrophotometer (Beckman DU640 UV/Vis Spectrophotometer) and the amylose content was calculated based on the standard curve. The samples were analyzed in triplicate.

Statistical analysis

All the analyses were done in triplicate and the data were statistically subjected to Analysis of Variance (ANOVA) using SPSS (IBM Statistics 21). Results are means of replicates (determined on a dry weight basis) ± standard deviation, significantly different at p <0.05.

RESULTS AND DISCUSSION

FTIR spectroscopy was used to verify the changes in the chemical structures of starch molecules resulting from acetylation and phthalation. The FTIR spectra of native, acetylated and phthalated cocoyam starches are presented in Figure 2, Figure 3, and Figure 4. In the spectrum of native starch, the peak at 3421.72 cm^{-1} and 2929.87 cm^{-1} correspond to O-H and C-H stretching, while the peaks at 1654.92 cm^{-1} and 1458.18 cm^{-1} correspond to O-H and C-H bending. Acetylated starches show new strong absorption bands at 1732.08 cm^{-1}; this indicates C=O stretching of acetyl group. **Mano, Koniarova and Reis (2003)** submitted a similar report. Phthalated starch showed new absorption bands at 1849.73 cm^{-1}, this new absorption indicates that phthalated starches were formed during the esterification process.

The FTIR spectra of native, acetylated and phthalated white yam starches are presented in Figure 5, Figure 6, and Figure 7. For the native starch, the peak at 3423.65 cm^{-1} and 2929.87 cm^{-1} correspond to O-H and C-H stretching, while the peaks at 1653.00 cm^{-1} and 1458.18 cm^{-1} correspond to O-H and C-H bending. Acetylated and the phthalated white yam starch did not show a new absorption band.

The FTIR spectra of native, acetylated and phthalated bitter yam starches are presented in Figure 8, Figure 9 and Figure 10. For native starch, the peaks at 3394.72 cm^{-1} and 2929.87 cm^{-1} correspond to O-H and C-H stretching, while the peaks at 1654.92 – 1637.56 cm^{-1} and 1438.18 cm^{-1} corresponds to O-H and C-H bending. Acetylated starches show new strong absorption bands at 1909.53 cm^{-1}; this indicates C=O stretching of acetyl group. Phthalated starch showed new absorption bands at 1703.14 cm^{-1} due to the carbonyl group of esters.

The results of the functional properties of native and modified starches are presented in Table 1. Native cocoyam starch has a water absorption capacity (WAC) of 8.70 ±0.02 g.g^{-1}, the value was increased after acetylation (9.37 ±0.15), and reduced after phthalation (6.50 ±0.30). The WAC values for native white yam and bitter yam starches were 8.17 ±0.15 and 8.94 ±0.05 g.g^{-1}, respectively, however, the values were increased (8.87 ±0.15, 8.50 ±0.01 g.g^{-1}) after acetylation and decreased (5.33 ±0.50 and 4.56 ±0.21 g.g^{-1}) after phthalation. Acetylated white yam starch showed the highest water absorption capacity while the phthalated bitter yam showed the least WAC. Acetylation increased the WAC of all the starches compared to their corresponding native starches, whereas phthalation decreased the WAC. A similar increase in the WAC upon acetylation was obtained in acetylated starches of sweet potato **(Lee and Yoo, 2009)** and corn **(Diop et al., 2011)**. The increase in the WAC in acetylated starches could be associated with the introduction of acetyl groups that impeded intermolecular chain associations, causing structural disorganization that facilitated water access in the amorphous region **(Xu, Dzenis and Hanna, 2005)**.

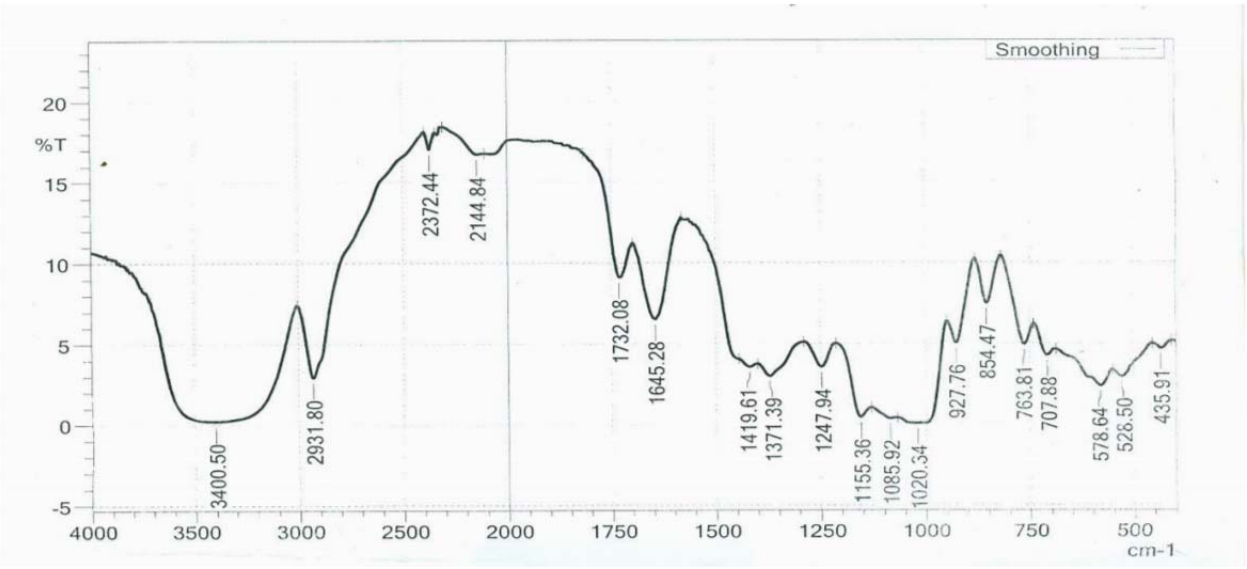

Figure 2 FTIR spectrum of native Cocoyam starch.

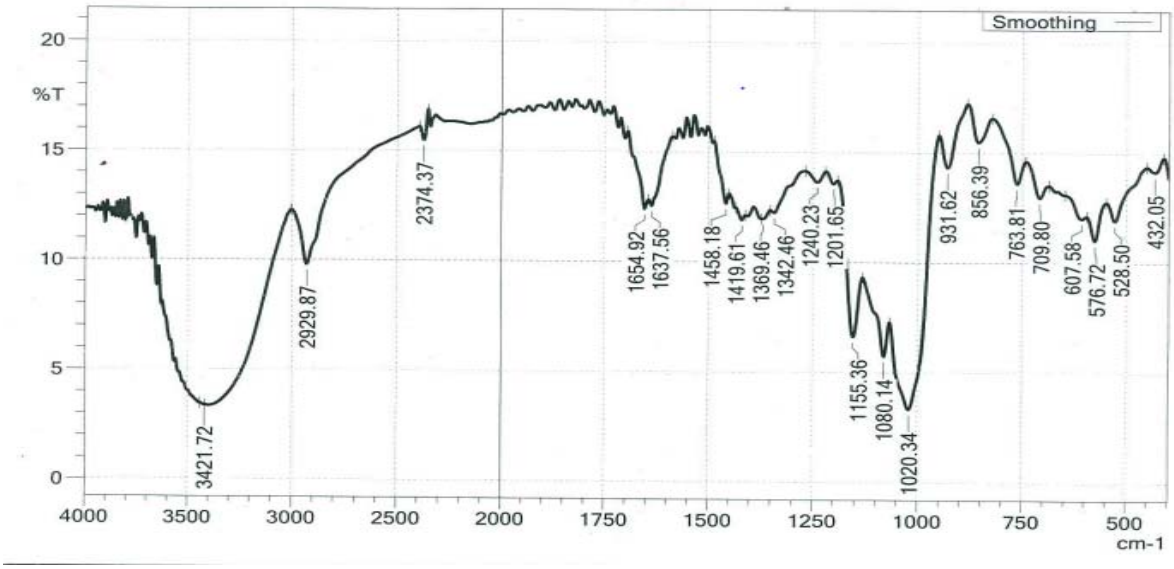

Figure 3 FTIR spectrum of acetylated Cocoyam starch.

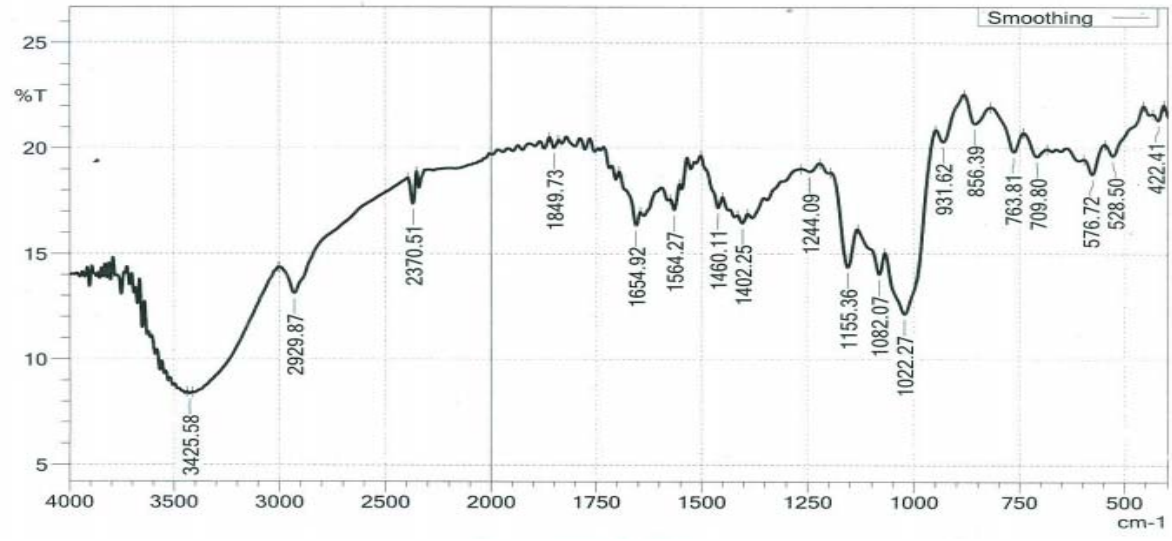

Figure 4 FTIR spectrum of phthalated Cocoyam starch.

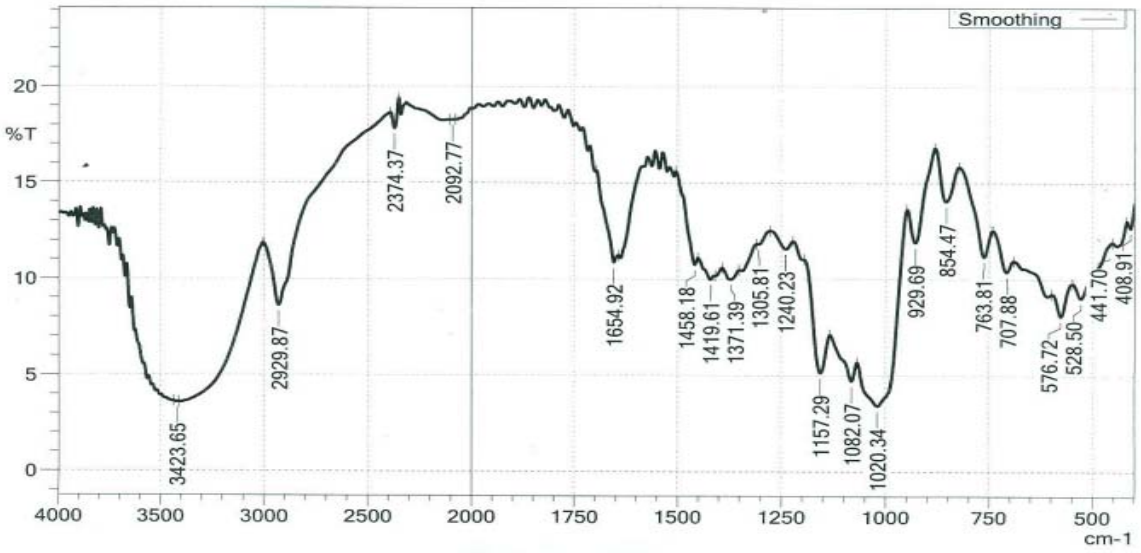

Figure 5 FTIR spectrum of native White yam starch.

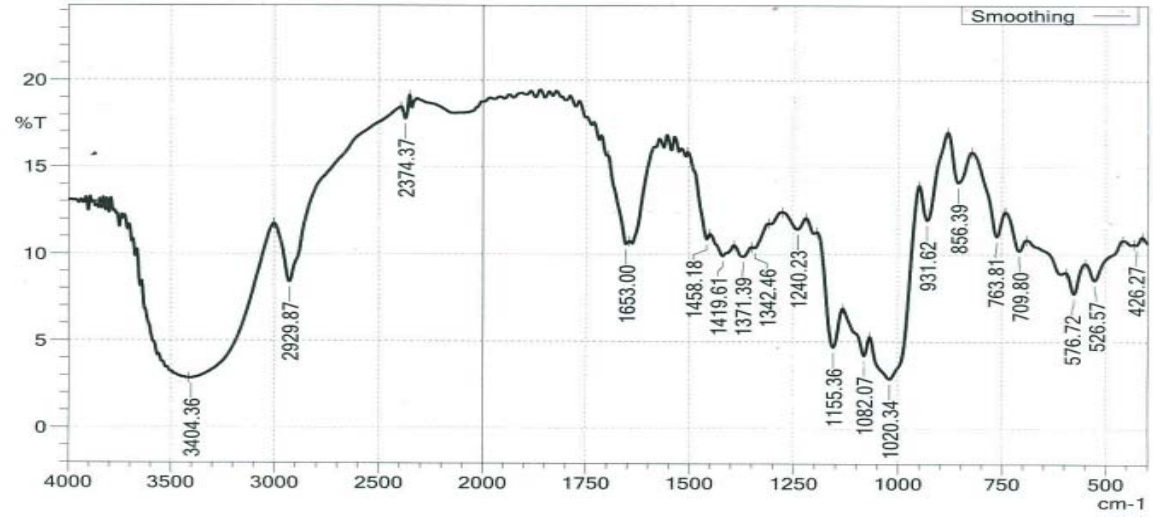

Figure 6 FTIR spectrum of acetylated White yam starch.

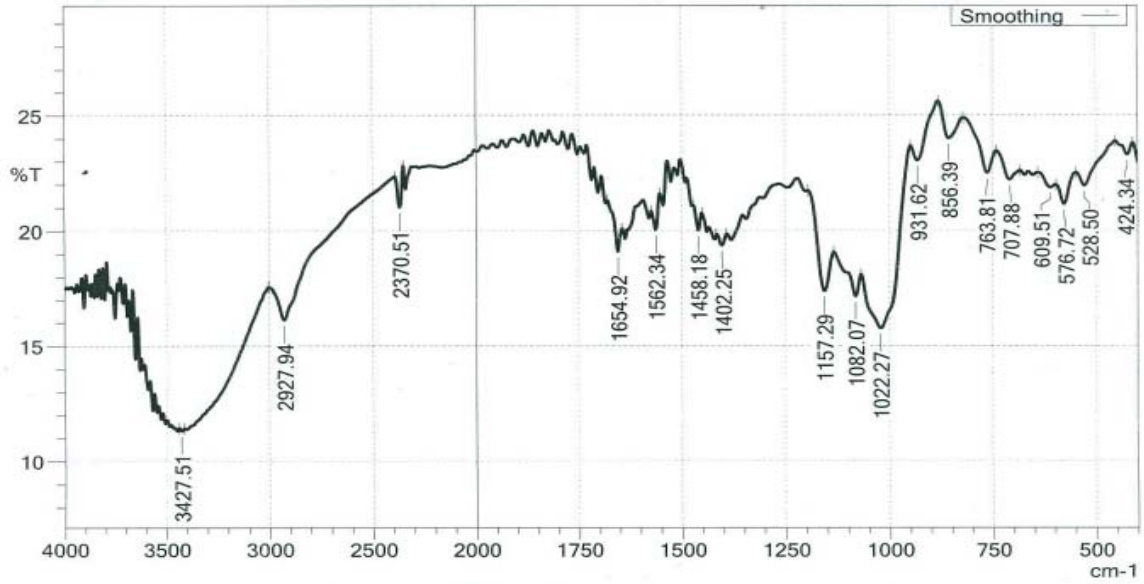

Figure 7 FTIR spectrum of phthalated white yam starch.

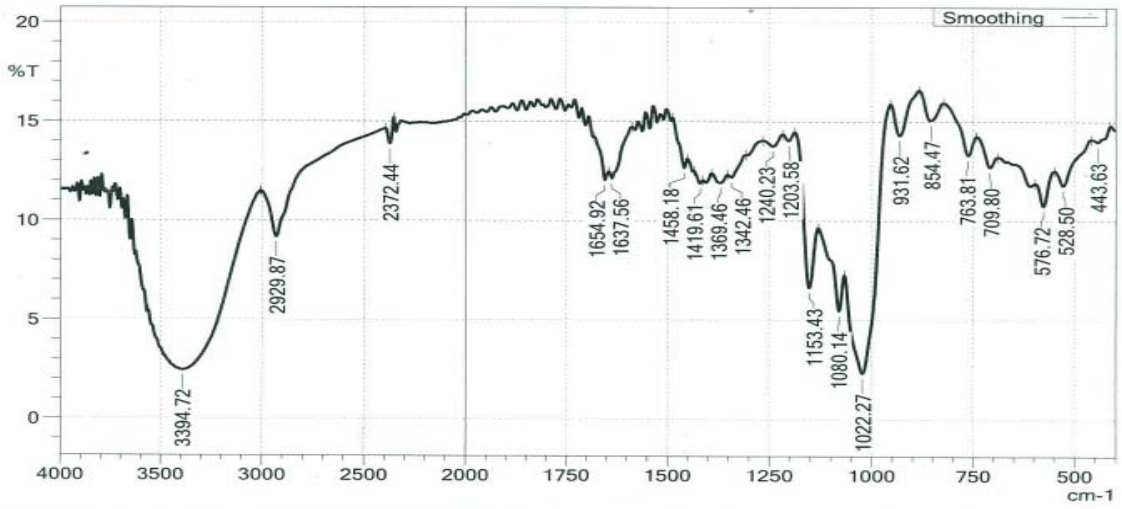

Figure 8 FTIR of sample native bitter yam starch.

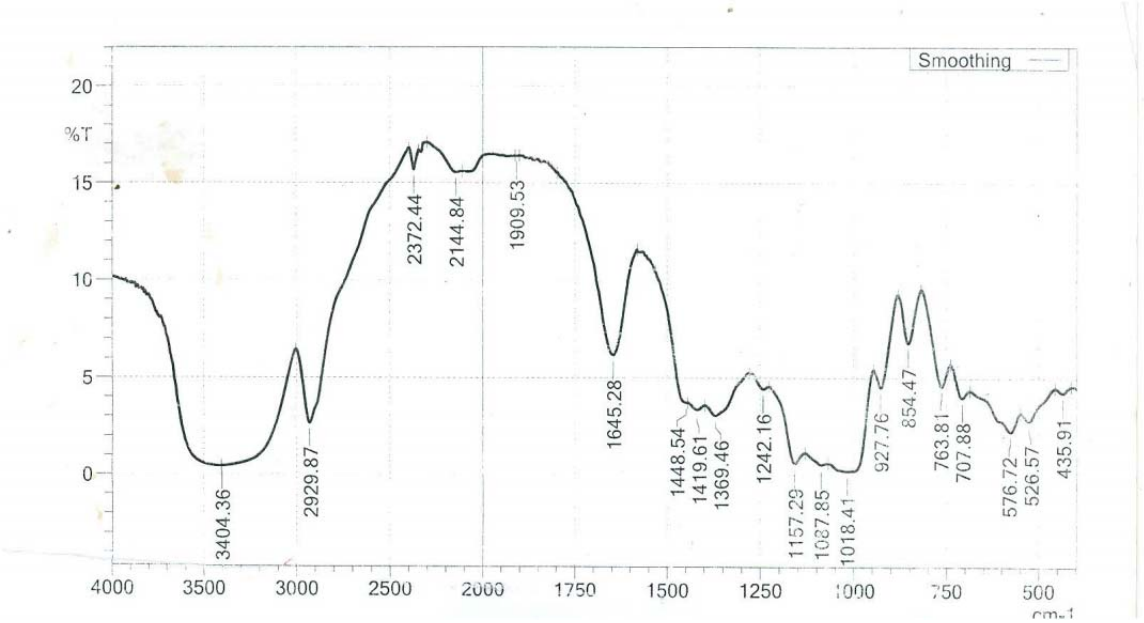

Figure 9 FTIR of acetylated bitter yam starch.

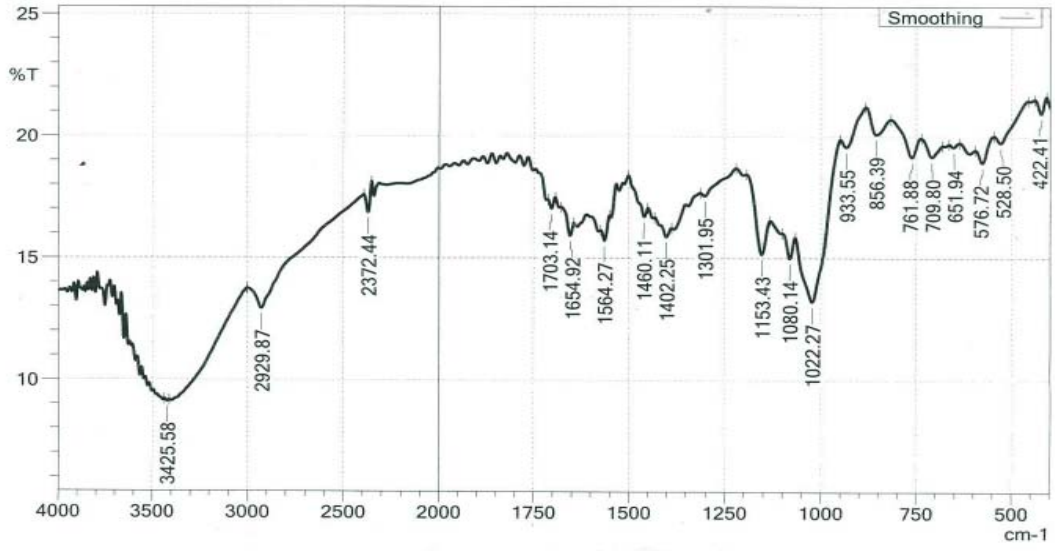

Figure 10 FTIR of sample phthalated bitter yam starch.

A decrease in WAC in phthalated starches could be that phthalation reinforces the structure of starch granules and limit water absorption which restricts the mobility of starch chain in the amorphous region **(Gunaratne and Corke, 2007)**. An increase in water absorption capacity of acetylated starches gave it the advantage of being used as a thickener in liquid and semi-liquids foods, it could be used in the development of confectionery products such as hard candies, the acetylated starches could also be used to produce absorbent materials such as disposable diapers and female napkins. The decreased water absorption capacity of phthalated starches suggests that they could be used in biodegradable films because of their reduction in hydrophilic property.

In the case of oil absorption capacity (OAC), Native cocoyam starch has an OAC of 2.57 ±0.21 which increased after acetylation (3.33 ±0.42 g.g-^{1}) and reduced after phthalation 1.63 ±0.25 g.g^{-1}. The OAC of the native white yam and bitter yam were 3.47 ±0.06 and 3.62 ±0.20 g.g^{-1}, respectively. However, the OAC values increased after acetylation and decreased after phthalation. Acetylated white yam starch showed the highest oil absorption capacity and phthalated cocoyam showed the least OAC. Acetylation increased the OAC of all the starches compared to their corresponding native starches, whereas phthalation decreased the OAC. This result suggested that acetylation enhanced the hydrophobic tendencies of the starches. A similar result was reported by **Uzomah and Ibe, (2011)**. WHO indicated that Acetylated starches had the strongest affinity for oil absorption. The obtained results showed that acetylation could be used to improve the oil absorption capacity of native starches.

Swelling power and solubility centre on the interaction between starch chains within the amorphous and crystalline regions, and the results are presented in Table 1. The swelling power of the native cocoyam, white yam, and native bitter yam starches increased after acetylation and decreased after phthalation. Acetylated bitter yam showed the highest swelling power while (4.63 ±0.15 g.g^{-1}) while phthalated white yam starch (1.64 ±0.04 g.g^{-1}) has the least swelling property. Increase observed in the swelling power of acetylated starches may be due to the weakening and disrupting of intra- and inter molecular hydrogen bonds in the starch chains, which may increase the accessibility of the starch granules to water. **(Lee and Yoo, 2009; Olu-Owolabi et al., 2014)**. Similar reports on swelling power after modifications have been documented **(Olayinka, Adebowale and Olu-Owolabi, 2013)**. The reduction in swelling power after phthalation could be linked to the possible structural disintegration within the starch matrix as a result of the modification. **Lawal (2004)**; **Adebowale and Lawal (2003)** reported that the lower swelling power of phthalated starches denotes the stability of starch granule. Starch swelling power is very important in the formulation of tablets and capsules, it is conceived that disintegrant works through swelling action **(Adebayo and Itiola, 1998)**. Consequently, starch with high swelling power is expected to release active pharmaceutical ingredients at a faster rate. Also, high swelling power leads to high digestibility which suggests improved dietary attributes **(Nuwamanya et al., 2010)**. The reduction in the swelling power of phthalated starches is an important property for their applications in biodegradable films.

Table 1 shows the water solubility of native and modified starches. Native cocoyam starch has a solubility of 1.56 ±0.06 g.g^{-1} which then increased after acetylation to 2.25 ±0.25 g.g^{-1}, and reduced after phthalation to 0.54 ±0.04 g.g^{-1}. The water solubility values for native white yam and bitter yam starches were 2.58 ±00.06 and 2.18 ±1.26, respectively. There was an increase in the values after acetylation, a reduction in value was however observed after phthalation. The increase in water solubility of acetylated form could be due to the structural rearrangement which weakens the granules and improves amylose leaching **(Lawal, 2004)**. Similar reports on water solubility on African yam bean and corn were submitted by **(Akintayo and Akintayo, 2009)** and **(Ayucitra, 2007)** starches. A decrease in water solubility after modification of Acha starch has been reported by **Olu-Owolabi et al. (2014)**.

The gelatinization temperatures of the native, acetylated and phthalated starches, are presented in Table 2. Acetylated and phthalated starches (cocoyam, whiteyam, and bitter yam) have lower gelatinization temperature compared to their corresponding native starches. These observations are in agreement with previous studies **(Lawal, 2011; Lee and Yoo, 2011)**. A decrease in gelatinization temperatures could be traced to the phthalation and acetylation processes in the starch polymer backbone, which permits improved flexibility **(Singh, Chawla and Singh, 2004)**. A decrease in gelatinization temperature is useful as a thickening agent in various industries, whereby the starch will form a gel at a lower temperature. Hoewever, thermal treatment reduced anti-nutritional agents **(Lauková et al., 2020)**.

The pH values for acetylated and phthalated starches were found to be slightly lower than their corresponding native starches, but still fall within the pH range of 3 − 9 obtained for most starches used in pharmaceutical, domestic, and food industries. The reduction in pH of native starches after acetylation and phthalation can be attributed to the modification processes thereby increasing the acidity of starch molecules. The amylose content of native cocoyam starch (20.90% ±0.06) was reduced (18.73% ±0.64) after acetylation, and increased after phthalation (30.31% ±0.17), amylose content of the native white yam(21.53% ±0.30) and bitter yam (22.73% ±0.31) decreased (18.63% ±0.17; 31.37% ±0.15) after acetylation, and increased (28.67% ±0.38; 27.53% ±0.38) after phthalation. Phthalated cocoyam starch showed the highest amylose content while acetylated white yam starch showed the least amylose content. The decrease in amylose content of acetylated starch was in agreement with the report of **Lawal (2004)**, on the reduction of amylose content of new cocoyam starch after acetylation. **Reddy, Haripriya and Suriya (2014)** also submitted a similar report on acetylated banana starch. Increased amylose contents of phthalated starches were in consonance with the report submitted by **Singh, Chawla and Singh (2004)** on modified potato and corn starches. Amylose content undergoes changes upon modification, also, structural differences between amylose and amylopectin can be considered as one of the most important factors of starch properties. Low amylose level makes starch a good source of food for diabetic and other

Table 1 Functional properties of native and modified starches.

Sample	WAC ($g.g^{-1}$)	OAC ($g.g^{-1}$)	SWP ($g.g^{-1}$)	Solubility ($g.g^{-1}$)	Gelation temp. (^oC)	Amylose content (%)	pH	Bulk density ($g.mL^{-1}$)	Amylopectin %
Native cocoyam sample	8.70 ±0.22^h	2.57 ±0.21^c	2.84 ±0.05^g	1.56 ±0.06^d	85	20.90 ±0.06^d	6.50	0.61 ±0.08^a	79.10 ±0.25^b
Acetylated yam sample	9.37 ±0.15^k	3.33 ±0.42^f	4.39 ±0.20^k	2.25 ±0.25^g	78	18.73 ±0.64^b	3.91	0.45 ±0.05^e	81.27 ±0.24^d
Phthalated cocoyam sample	6.50 ±0.30^c	1.63 ±0.25^a	1.84 ±0.05^c	0.54 ±0.04^a	80	30.31 ±0.17^k	5.72	0.32 ±0.06^d	69.69 ±0.05^e
Native white yam	8.17 ±0.15^e	3.47 ±0.06^h	2.15 ±0.05^d	2.58 ±0.06^h	82	21.53 ±0.30^e	6.20	0.65 ±0.22^h	78.47 ±0.12^c
Acetlated white yam	8.87 ±0.15^b	4.53 ±0.06^l	3.87 ±0.08^j	3.00 ±0.08^i	78	18.63 ±0.17^b	4.52	0.69 ±0.03^i	81.37 ±0.21^f
Phthalaled white yam	5.33 ±0.50^a	3.37 ±0.21^g	1.64 ±0.04^b	1.50 ±0.16^c	80	28.67 ±0.38^j	5.67	0.53 ±0.08^f	71.33 ±0.32^g
Native bitter yam	8.94 ±0.05^j	3.62 ±0.20^i	3.70 ±0.25^i	2.18 ±1.26^f	82	22.73 ±0.31^f	6.75	0.51 ±0.04^c	77.27 ±0.51^b
Acetylated bitter yam	8.50 ±0.27^g	4.03 ±0.06^k	4.63 ±0.15^l	3.24 ±0.17j	74	21.37 ±0.15^g	4.61	0.43 ±0.03^b	78.63 ±0.22^d
Phthalated bitter yam	7.56 ±0.21^d	2.23 ±0.15^b	2.61 ±0.15^e	1.56 ±1.24^d	80	27.53 ±0.38^i	5.22	0.34 ±0.07^k	72.47 ±0.05^h

Note: Values are means of three replicates (determined on dry weight basis) ± standard deviation, significantly different at $p < 0.05$. WAC – water absorption capacity; OAC – oil absorption capacity; SWP – swelling power.

Table 2 Least gelation concentration of native and modified starches.

Sample	2%	4%	6%	8%	10%	12%	14%	16%
Native cocoyam sample	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Acetylated yam sample	-Viscous	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Phthalated cocoyam sample	-Viscous	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Native white yam	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Acetlated white yam	-Viscous	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Phthalaled white yam	-Viscous	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Native bitter yam	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Acetylated bitter yam	-Viscous	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel
Phthalated bitter yam	-Viscous	-Viscous	-Viscous	+ Gel	+ Gel	+ Gel	+ Gel	+ Gel

Note: Determination were carried out in triplicates. (-) No gelation and (+) gelation.

health conscious beings **(Agbo and Odo, 2010)**. However, high amylose content often leads to retrogradation.

Table 1 shows the bulk density of native and modified starches. Native cocoyam starch has a bulk density of 0.61 ±0.08 $g.g^{-1}$, which decreased after both acetylation and phthalation to 0.45 ±0.05 and 0.32 ±0.06 $g.g^{-1}$. The bulk density values of the native white yam and bitter yam starches (0.65 ±0.22 and 0.51 ±0.04 $g.g^{-1}$) decreased after both acetylation (3.00 ±0.08 and 3.24 ± 0.17 $g.g^{-1}$,) and phthalation (1.50 ±0.16 and 1.56 ±1.24 $g.g^{-1}$). Native white yam starch has the highest bulk density of 0.65 ±0.22 $g.g^{-1}$, while phthalated cocoyam starch (0.32 ±0.06) has the least bulk density. Acetylation and phthalation reduced the bulk density of the starches. The higher bulk density of a material, the more the quantity which can be packaged in a confined space **(Fagbemi, 1999)**. Materials with high bulk density are regarded as heavy.

The results of the least gelation of native and modified starches are presented in Table 2. The lowest gelation concentration for native cocoyam, white yam, and bitter yam starches was 6%. However, none of the starches showed positive results at the concentrations of 2 and 4%. At 8% concentration, all the native and modified starches formed a gel, all other higher concentrations showed positive results. It was observed that an increase in concentration leads to gel formation. A similar increase in the least gelation concentration upon acetylation was obtained in acetylated starches of African yambean starch **(Akintayo and Akintayo, 2009)** and sweet potato starch **(Diop et al., 2011)**. Thus, the results suggested that the native starches are better gelating food additives than acetylated and phthalated starches.

CONCLUSION

It can be concluded that phthalation of native starches reduced water absorption capacity, swelling capacity, solubility, oil absorption capacity, swelling power, amylose content of the starches which are better properties of biodegradable polymers while acetylation increased

water absorption capacity, oil absorption capacity, swelling power and solubility of the starches which make the starches to be useful in confectioneries. This study, apart from establishing the characterization differences between native and modified starches has also provided information that the modified starches have more and improved applications in food industries.

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SPONTANEOUS FERMENTATION IN WINE PRODUCTION AS A CONTROLLABLE TECHNOLOGY

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ABSTRACT

This study focuses on the isolation of a consortium of microorganisms from spontaneously fermenting must that naturally contain lactic acid bacteria, non-saccharomyces yeasts, and saccharomyces yeasts. To collect the greatest diversity of microorganisms, the consortium was taken from the point of micro-sparkling. Based on the growth curves, isolation was performed using individual special nutrient media, and the isolates were subsequently multiplied in the nutrient medium. Individual isolates were then used for fermentation tests to monitor the percentage of fermented sugar and hydrogen sulphide production. The highest fermentation abilities were achieved in the isolates containing *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. The smallest amount of ethanol was formed from the isolates containing *Hanseniaspora uvarum*, while *Candida sake* isolate produced the lowest amount of hydrogen sulphide and *Zygosaccharomyces bailii* produced the highest. The other isolates produced an average amount. Based on these results, a consortium containing the given isolates in a certain ratio was compiled.

Keywords: spontaneous fermentation; yeast cultivation; yeast isolation; growth curves

INTRODUCTION

Saccharomyces cerevisiae (*S. cerevisiae*) is a type of yeast that performs alcohol fermentation and is widely used as a fermentation starter. During the alcoholic fermentation of grape must, *S. cerevisiae* becomes the dominant species with the increasing ethanol concentration **(Mas, Guillamón and Beltran, 2016)**. Therefore, the isolation of natural *S. cerevisiae* is generally carried out from must be fermented by spontaneous fermentation **(Versavaud et al., 1995; Valero et al., 2007; Clavijo, Calderón and Paneque, 2010; Cordero-Bueso et al., 2011; Viel et al., 2017; Crosato et al., 2018)**, suggesting that *S. cerevisiae* is common on grapes. Recently, **Taylor et al. (2014)** reported that *Saccharomyces* sp. makes up less than 0.00005% of the yeast population on ripe grapes.

Similarly, **Fleet (2003)** reported its presence at concentrations below 10 – 100 cfu.g^{-1}, and **Fleet (2003)** and **Martini, Ciani and Scorzetti (1996)** reported that the total number of cells never exceeds 10 cfu.g^{-1} on grape berries (cfu - colony forming units).

The diversity and quantity on grapes always vary depending on the variety, stage of ripening, terroir, vintage, vineyard age, soil type, the geographical location of the vineyard, climatic conditions, diseases, pests, and vineyard work used **(Pretorius, 2000; Mannazzu, Clementi and Ciani, 2002; Valero et al., 2007; Barata, Malfeito-Ferreira and Loureiro, 2012; Setati et al., 2012; Bokulich et al., 2014)**.

Equally important is the secondary microbial process of malolactic fermentation (MLF), which is when malic acid is converted into nicer-tasting lactic acid. Lactic acid bacteria (LAB) are involved in this process **(Bauer and Dicks, 2004)**, and this microbial development is also associated with the release of other metabolites that are capable of affecting wine quality **(Campbell-Sills et al., 2016)**.

Model microorganisms for alcoholic and malolactic fermentation are *Saccharomyces cerevisiae* and *Oenococcus oeni*. The selected strains belonging to these two species have been used to design starter cultures that are useful for promoting alcoholic fermentation (AF) and MLF, thus making the fermentation process more manageable **(Garofalo et al., 2016; Berbegal et al., 2017; Petruzzi et al., 2017)**.

MLF can also occur spontaneously, but its course is often unpredictable. It can take place over several months after the end of AF or fail due to unfavorable conditions in a wine based on the wine's pH, ethanol, and SO_2 content **(Berbegal et al., 2016; Lucio et al., 2017)**.

Another risk of spontaneous MLF is the formation of undesirable by-products, colour reduction, and higher synthesis of biogenic amines **(Guo et al., 2015; Henríquez-Aedo et al., 2016)**.

Oenococcus oeni is generally the main species used as an MLF starter due to its easy adaptation to wine conditions. New strains such as *L. plantarum* are currently being discovered, however, that can also survive and adapt well to the viticulture. These strains also have more favorable biological properties compared to *O. oeni*, such as higher growth rate, creation of a more complex aromatic profile, and the prevention of undesirable by-product formation **(du Toit et al., 2011; Brizuela et al., 2017)**.

Therefore, the aim of this study was to isolate yeast and lactic bacteria from the vineyard and carry out a fermentation experiment using these isolates.

Scientific hypothesis

Each vineyard has its own wine of microbiota, which affects the quality of the resulting wine. Separation and cultivation methods can be used to characterization and multiplying individual microorganisms. Based on the fermentation and H_2S production tests, the resulting consortium can be assembled, which can be used for fermentation, thereby promoting both the fermentation process and the terroir of the wine.

MATERIAL AND METHODOLOGY

The aim was to obtain a functional consortium of wine microorganisms that was characterized in terms of biotechnology and taxonomy. This consortium was separately cultivated to achieve the required amounts and then used to inoculate a drained batch of Hibernal must in which fermentation and H_2S production were monitored.

Materials

MEA+T Malt Extract Agar with Tetracycline (a broad-spectrum antibiotic against both gram-positive G+ and gram-negative G- bacteria). Only eukaryotic microorganisms grow on this medium.

Composition: agar – 15 $g.L^{-1}$, malt extract – 30 $g.L^{-1}$, mycological peptone – 5 $g.L^{-1}$, (manufacturer: Sigma Aldrich)

WLN: Wallerstein Nutrient Agar for counting and culturing yeast and bacteria.

Composition: agar – 20 $g.L^{-1}$, bromocresol green – 0.022 $g.L^{-1}$, calcium chloride – 0.125 $g.L^{-1}$, casein enzymic hydrolysate – 5 $g.L^{-1}$, dextrose – 50 $g.L^{-1}$ (manufacturer: Sigma Aldrich).

MRS (Agar according to DeMan, Rogosa, and Sharpe): Nutrient agar for the determination of lactic bacteria.

Composition: Agar – 12 $g.L^{-1}$, diammonium bicarbonate – 2 $g.L^{-1}$, potassium hydrogen phosphate – 2 $g.L^{-1}$, D (+) – glucose – 20 $g.L^{-1}$, magnesium sulphate – 0.1 $g.L^{-1}$, manganese sulphate – 0.05 $g.L^{-1}$, meat extract – 5 $g.L^{-1}$, sodium acetate – 5 $g.L^{-1}$, universal peptone – 10 $g.L^{-1}$, yeast extract – 5 $g.L^{-1}$. (manufacturer: Sigma Aldrich)

YPD (Yeast extract Peptone Dextrose) agar: Solid medium for yeast multiplication.

Composition: Bacteriological peptone – 20 $g.L^{-1}$, yeast extract – 10 $g.L^{-1}$, glucose – 20 $g.L^{-1}$, agar – 15 $g.L^{-1}$. (manufacturer: Sigma Aldrich)

ME (M-enterococcus) agar: Agar consists of tryptose; yeast extract; glucose; disodium hydrogen phosphate; sodium azide; 2, 3, 5-triphenyltetrazolium chloride; agar; and distilled or deionized water. (manufacturer: Sigma Aldrich)

BIGGY (Bismuth Sulphite Glucose Glycine Yeast Agar): Selective and differential medium with addition of bismuth salt for H_2S detection. Bismuth reacts with the resulting sulfane to form a precipitate that colours the agar below the colony.

Composition: Glucose – 10.0 $g.L^{-1}$, glycine – 10.0 $g.L^{-1}$, bismuth ammonium citrate – 5.0 $g.L^{-1}$, sodium sulphite – 3.0 $g.L^{-1}$, yeast extract – 1.0 $g.L^{-1}$, agar – 13.0 $g.L^{-1}$. (manufacturer: Sigma Aldrich)

The must of the Hibernal variety: This variety was developed in Germany in 1944 as a hybrid of Seibel 7053 (Chancellor) and Riesling. The must be clarified by sedimentation (after 24 hours). The turbidity value of the must after clarifying was approx. 400 NTU and this was not adjusted. The sugar content was 16 °NM, pH was 3.51, titratable acid content was 6.48 $g.L^{-1}$, and assimilable nitrogen content was 321 $mg.L^{-1}$.

Isolation of yeasts

The must of the Hibernal variety was fermented spontaneously. During this spontaneous fermentation, 20 mL of the matrix (must, fermentation must, wine) was taken at selected monitoring points (must, micro-sparkling, 4 and 8 vol. % ethanol, end of fermentation). The sample obtained was subsequently diluted using the so-called decimal series. From each dilution, 250 mL was pipetted onto Petri dishes with MEA+T, WLN, and MRS culture medium, and a microbiological rod smear was performed. The Petri dishes were then placed in a thermostat (30 °C; WLN and MEA+T – 3 days; MRS – 7 days).

At the end of the cultivation, the total number of microorganisms and individual colonies was enumerated. Based on a combined analysis of phenotypic characters (macroscopic and microscopic properties), a sampling point was selected (the most suitable was the micro-sparkling point - the diversity of technologically important microorganisms was evaluated – saccharomyces and non-saccharomyces yeasts and lactic bacteria) to serve as a source for consortium acquisition.

Determination of growth characteristics of individual isolates of the 2018 Wine Microorganism Consortium (growth curves)

The aim was to determine the growth characteristics of individual isolates. Individual isolates of microorganisms were pre-cultured in standard media (yeast – YPD; lactic acid bacteria - MRS) with the following culture conditions: 30 °C; shaking 120 rpm; yeast 24 h; and lactic acid bacteria 72 h. The obtained cell suspensions were centrifuged (10 mins; 10 °C; 10,000 rpm), washed with saline solution, and then resuspended in the selected media (yeast – YPD, ME, YPD_{mod}; lactic acid bacteria – MRS, YPD, and YPD_{mod}) so that the resulting optical density value of the suspension was 0.2 at a wavelength of 600 nm.

The obtained suspension was then pipetted onto Bioscreen C culture plates (Oy Growth Curves Ab Ltd). Each arrangement (microorganism x medium) consisted of five repetitions to ensure the achievement of relevant results.

Table 1 Results of the operational microbiological monitoring of the fermentation process.

Sampling time interval	MEA+T (cfu.mL^{-1})	WLN (cfu.mL^{-1})	MRS (cfu.mL^{-1})
Must	3.80E +05	4.00E +05	4.00E +02
Micro-sparkling	1.75E +06	2.35E +06	7.00E +03
EtOH 4 Vol. %	8.00E +06	5.00E +06	5.00E +02
EtOH 8 Vol. %	9.00E +06	1.20E +07	1.20E +02
End of fermentation	5.00E +04	4.00E +04	0

Note: cfu - colony forming units.

The culture conditions of the Bioscreen C device were set as follows: 30 °C; shaking every 3 mins; duration of one shaking cycle = 1 min; and the so-called wide band of wavelengths – WB (420 – 620 nm). The maximum growth rate μ (h^{-1}) was then calculated from the measured data, and the maximum optical density OD_{MAX} was determined. These data, along with the course of growth curves, served to assign culture media to individual isolates.

Identification of microorganisms

The MALDI TOF (matrix-assisted laser desorption/ionization coupled with time of flight mass spectrometry) method was used to identify microorganisms. It is a very accurate, simple method, able to determine high molecular weight substances, proteins, peptides, lipids, nucleic acids, carbohydrates **(Huong et al., 2014)**.

An essential part of the MALDI TOF measurement was the preparation of fresh α-cyano-4-hydroxycinnamic acid. The organic solvent was prepared by mixing 500 μL of acetonitrile (100%), 475 μL of distilled water, and 25 μL of trifluoroacetic acid. Before use, 250 μL of organic solvent was added to the plastic tube. The contents of the tube were vortexed until the complete dissolution of the crystals. α-cyano-4-hydroxycinnamic acid was stored in the dark place and its preparation is ideal the day before the measurement.

The cultures were applied to the clean metal plate for MALDI TOF and the culture was allowed to dry on the plate. It was then covered with 1 microliter of α-cyano-4-hydroxycinnamic acid. At the same time, it was important to homogenize the sample and matrix **(Jarolímková, 2017)**.

Unlike the analysis of bacteria, preprocessing of the yeast isolates was required to extract fungal proteins. The protein extraction method used to process yeast isolates for MALDI-TOF MS was adapted directly from established methods used to identify difficult bacterial isolates. Specifically, 1 to 5 colonies of an isolate were inactivated in 75% ethanol, pelleted, and then suspended in a 1:1 mixture of 70% formic acid and acetonitrile. The resulting supernatant was then analyzed by MALDI-TOF MS **(Marklein et al., 2009; Bader et al., 2011; Dhiman et al., 2011)**. The results of the identifications are in Table 5.

Optaining pure cultures: Separation and lyophilisation

This procedure aimed to cultivate individual isolates of technologically important microorganisms and preserve them using the lyophilization method. Separate cultivation of individual microbial isolates was performed based on the information obtained from the growth characteristics. Individual taxa were first pre-cultured in 250 mL Erlenmeyer flasks (100 mL medium volume; orbital stirring 120 rpm; 20 °C). The media and the culture times are shown in (Table 3). The pre-cultured cell suspension was examined microscopically (cell morphology, elimination of contamination) and centrifuged (10,000 rpm; 10 mins; 10 °C).

After separating the supernatant, the pellet was washed with saline solution and re-centrifuged (10,000 rpm; 10 mins; 10 °C) and resuspended in the pure culture medium. The prepared suspension served as the inoculum for the second cultivation stage, which was carried out in 2,000 mL Erlenmeyer flasks (1,000 mL medium volume; orbital stirring 100 rpm; 30 °C). The media and the culture times used for the individual isolates are shown in (Table 5).

After the cultivation was complete, the suspension was repeatedly centrifuged and washed as described in the previous step. The obtained biomass was mixed with cryoprotective medium and shock-frozen (70 °C; 24 h). The frozen suspension was then lyophilized. The viability of the obtained dehydrated biomass was then determined, and according to the qualitative and quantitative microbiological analysis (Table 2) and the cell viability in the lyophilisate, the 2018 Wine Microorganism Consortium was compiled.

Fermentation tests using a consortium

Fermentation tests

Individual yeast isolates were initially cultured in 50 mL Erlenmeyer flasks (25 mL medium volume; orbital stirring 120 rpm; 30 °C). The media and the culture times used for the individual isolates are shown in (Table 5). In the obtained cell suspension, the cell density was determined by microscopic cell counting in a so-called Bürker chamber. The calculated amount of this suspension was then pipetted to a final concentration of 10^8 cells.mL^{-1} in a 250 mL Erlenmeyer flask (100 mL YPDm medium volume; without shaking; 25 °C). Fermentation was monitored by the gravimetric method, and weight loss due to the metabolic conversion of fermentable sugars to carbon dioxide and ethanol was observed.

H_2S production

Individual isolates were inoculated onto a BIGGY agar identification medium using a microbiological loop. Individual Petri dishes were statically cultured at 30 °C for 3 days. Based on the visual evaluation, the individual isolates were marked as low, medium, and high producers of H_2S.

BiGGY, Bismuth Sulphite Glucose Glycine Yeast Agar, is based on the formulation developed by **Nickerson (1953)** and mainly used for the isolation and presumptive identification of *Candida* species. In a study of sulphite

reduction by yeasts, the ability of many yeasts to reduce a bismuthyl hydroxy polysulphite was noted. Growth on an acidic or neutral medium containing bismuth sulphite produced black colonies because of the extra-cellular reduction of the bismuth sulphite, to bismuth sulphide. The bismuth sulphite complex confers a high degree of selectivity to the medium, and most strains of bacteria are inhibited on BIGGY Agar. In this study, BIGGY agar was used as a simple and rapid method to compare the rate of H_2S production between pure yeast isolates.

Statistical analysis

Statistical analyses and figures were generated using Excel 2007 software packages (manufactured by Microsoft Office, USA) and Statistica 10 statistical software (Copyright © StatSoft). The Statistica 10 software was used to process growth curves data and create their line graphs.

RESULTS AND DISCUSSION

Isolation of yeasts

The results of the microbiological analysis are shown in (Table 1). These data are comparable with the normal course of fermentation of the grape must. Based on the phenotypic analysis, a consortium was selected from the point of micro-sparkling.

The quantitative parameters of the individual taxa of the 2018 Wine Microorganism Consortium are given in (Table 2). The individual values in (Tables 1 and Table 2) were averaged from three measurements.

Through the application of microbiological techniques, the 2018 Wine Microorganism Consortium was obtained from the spontaneous batch, which was characterized in qualitative and quantitative terms.

Growth curves

The growth curve courses are shown in (Figures 1 and Figure 2), while the numerical parameters of the growth characteristics are shown in (Tables 3 and Tables 4). The growth characteristics of individual isolates were determined based on the growth curves of different types of media. These characteristics were used to assign culture media and culture times to individual isolates (Table 3).

Obtaining pure cultures: Separation and lyophilisation

To culture and lyophilise the 2018 Wine Microorganism Consortium, 30 g of the consortium was prepared and used for fermentation for 100 litres of must. The representation of the individual isolates can be seen in Table 4.

Table 2 Quantitative parameters of isolated taxa of the 2018 Wine Microorganism Consortium. Sampling from the point of micro-sparkling.

Isolate marking	Determined cell concentration (cfu.mL^{-1} of matrix)
LAB01	5.00E+03
LAB02	2.00E+03
Y01	3.50E+05
Y02	2.00E+05
Y03	1.20E+06
Y04	2.00E+05
Y05	1.00E+05
Y06	9.50E+05
Y07	1.10E+06

Note: cfu – colony forming units.

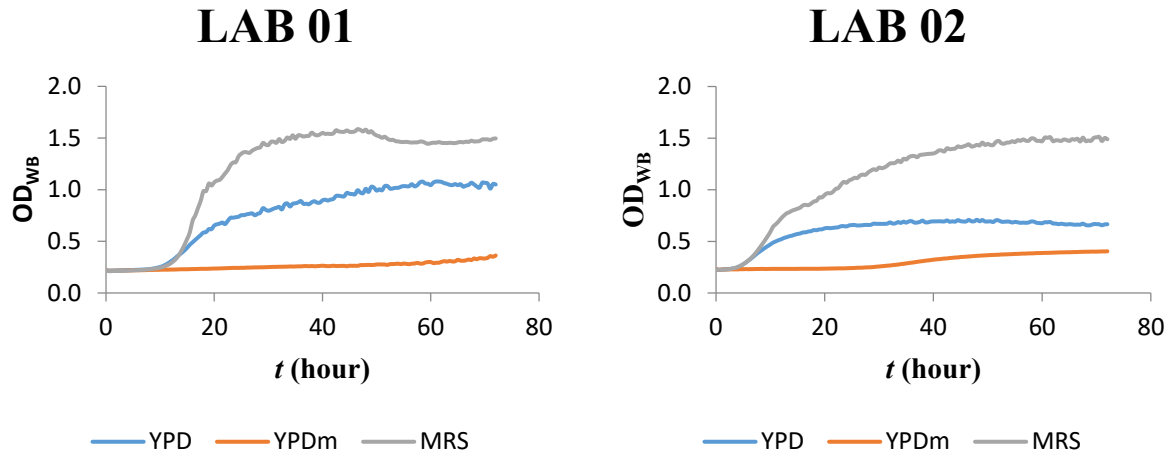

Figure 1 Growth curves of isolates LAB01 and LAB02; lactic acid bacteria.

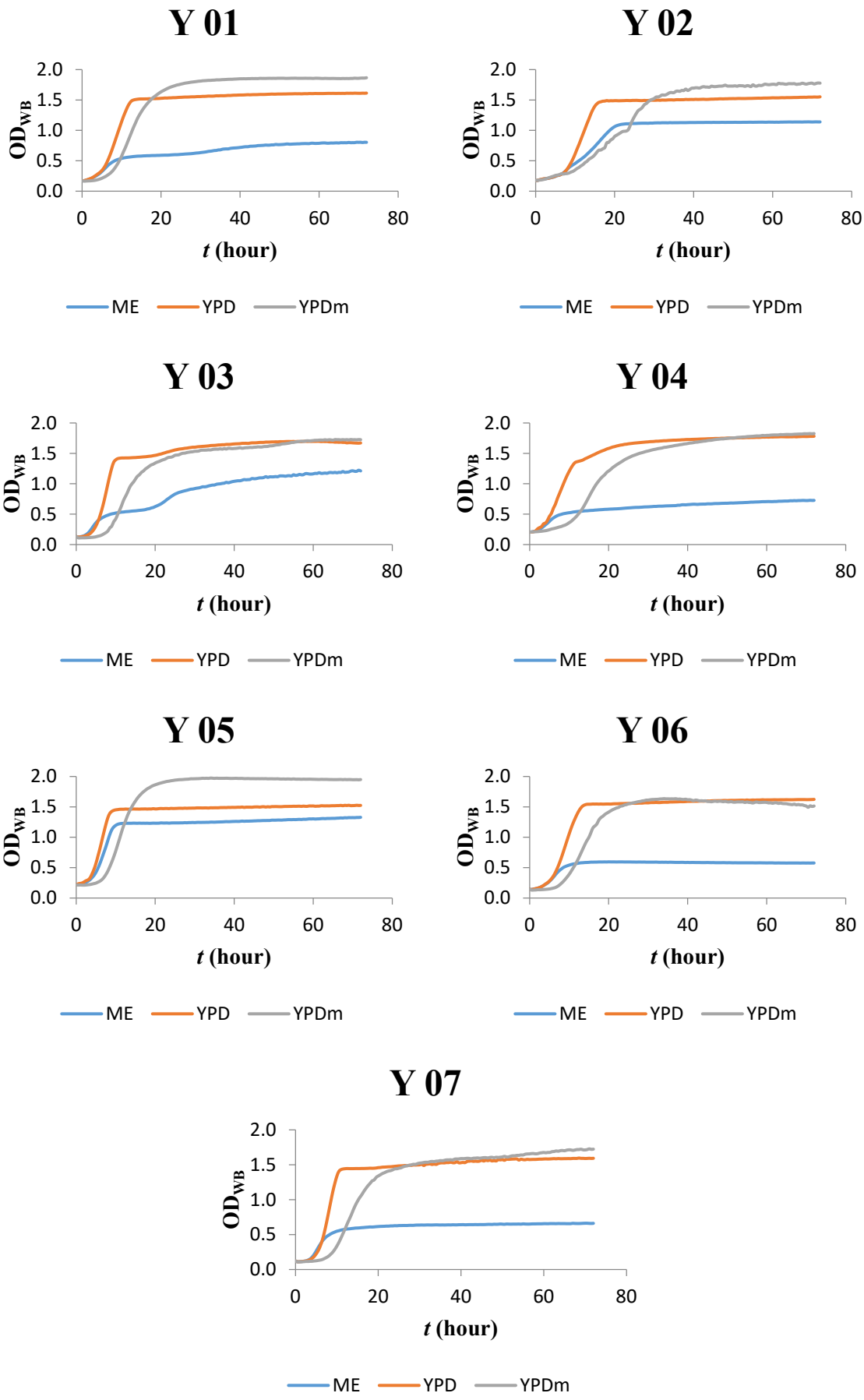

Figure 2 Growth curves of isolates Y01 – Y07; yeasts.

Table 3 Assignment of culture media and culture times to individual isolates.

Isolate marking	Culture medium	Cultivation time (h)
LAB01	MRS	32
LAB02	MRS	48
Y01	YPDm	22
Y02	YPDm	36
Y03	YPD	9
Y04	YPD	17
Y05	YPDm	24
Y06	YPD	12
Y07	YPD	10

Table 4 Weight composition of lyophilised 2018 Wine Microorganism Consortium preparation.

Isolates	Taxonomic identification
LAB01	*Lactobacillus brevis*
LAB02	*Lactobacillus plantarum*
Y01	*Hanseniaspora gulliermondi*
Y02	*Saccharomyces cerevisiae 1*
Y03	*Hanseniaspora uvarum 1*
Y04	*Hanseniaspora uvarum 2*
Y05	*Saccharomyces cerevisiae 2*
Y06	*Zygosaccharomyces bailii*
Y07	*Candida sake*

Table 5 Identification of individual isolates in the Consortium of Wine Microorganisms, 1 and 2 are different axenic cultures.

Isolates	Taxonomic identification
LAB01	*Lactobacillus brevis*
LAB02	*Lactobacillus plantarum*
Y01	*Hanseniaspora gulliermondi*
Y02	*Saccharomyces cerevisiae 1*
Y03	*Hanseniaspora uvarum 1*
Y04	*Hanseniaspora uvarum 2*
Y05	*Saccharomyces cerevisiae 2*
Y06	*Zygosaccharomyces bailii*
Y07	*Candida sake*

Table 6 Numerical parameters of fermentation tests of yeast isolates.

Isolate marking	Max. ethanol production rate (vol. %/day)	Max. EtOH concentration achieved (vol. %)
Y01	0.375	7.27
Y02	2.530	13.28
Y03	0.900	6.98
Y04	0.167	2.98
Y05	1.992	13.30
Y06	1.017	9.37
Y07	0.492	7.20

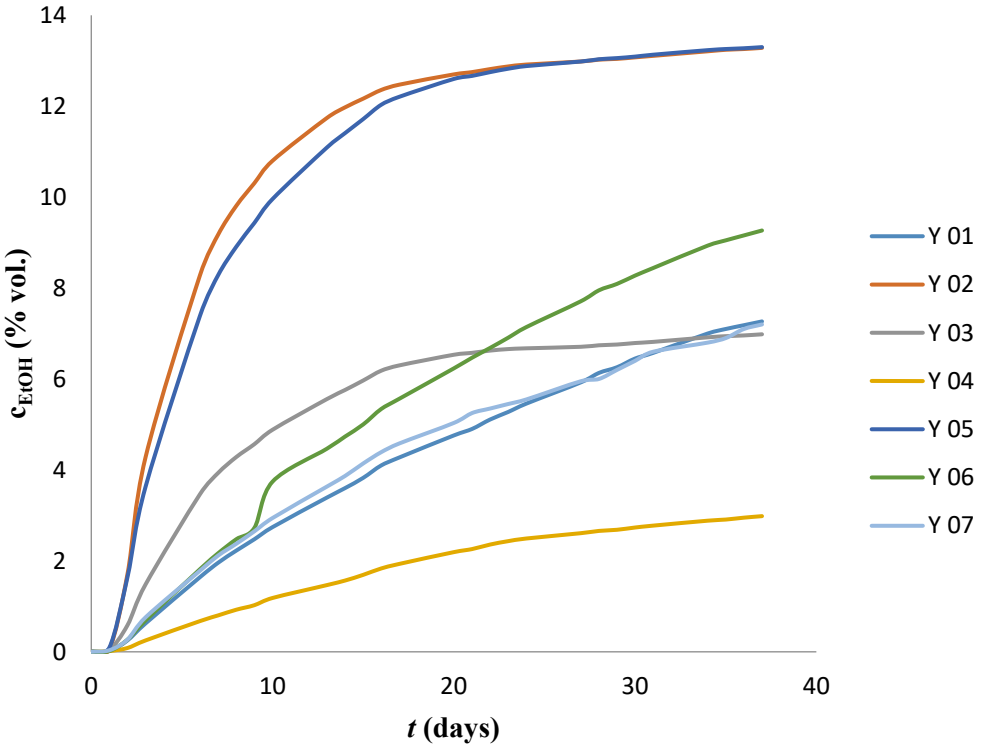

Figure 3 Course of fermentation tests of yeast microorganisms from the 2018 Wine Microorganism Consortium.

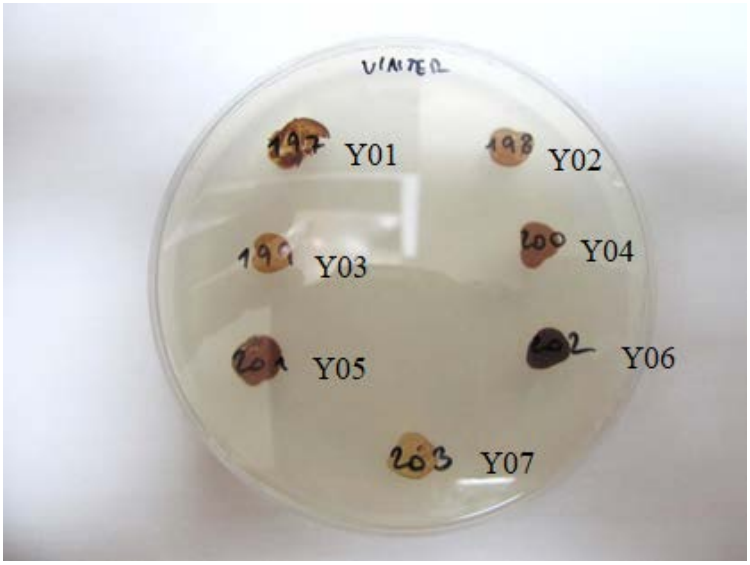

Figure 4 H_2S production by isolates Y01, Y02, Y03, Y04, Y05, Y06, and Y07.

Table 7 Evaluation of H_2S production by individual yeast isolates.

Isolate marking	H_2S production
Y01	Medium
Y02	Medium
Y03	Medium
Y04	Medium
Y05	Medium
Y06	High
Y07	Low

The growth curves, fermentation tests, and H_2S production results were used to inform the composition of the consortium so that the entire mixture exhibited the best fermentation capabilities, including the desired strains of lactic acid bacteria. Isolates Y03, Y06, and Y07 had the largest mass representation as characterized by a looser fermentation process but had the ability to ferment alcohol to 6.98, 9.37, and 7.20% vol. alc., respectively. These isolates showed low, medium, and high H_2S-producing ability. Isolates Y02 and Y05 had the second largest mass representation of the resulting consortium as characterized by high sugar fermentability up to an alcohol content of 13.28 and 13.30% vol. alc., respectively. The fermentation process was not gradual, however, which could negatively affect the quality of the wine. Both isolates showed mean H_2S production capacity. The isolate Y04 had a low mass representation. It showed the lowest sugar fermentability to an alcohol content of only 2.98 and had a medium ability to produce H_2S.

Table 5 contains the identification of individual isolates. The consortium consists of 2 strains of lactic acid bacteria and 7 strains of saccharomyces and non-saccharomyces yeast. The most abundant yeast strains of Consortium are *Hanseniaspora uvarum*, *Zygosaccharomyces bailii* and *Candida sake.*

Fermentation using isolates

Fermentation tests

Based on the results of the fermentation tests (Figure 3 and Table 6), we can conclude that there was some similarity between isolates from the 2018 Wine Microorganism Consortium. For example, between isolates Y02 and Y05 and isolates Y01 and Y07. For the Y03 and Y06 strains, there was always a certain deviation that distinguished them from the other isolates, and isolate Y04 showed a completely different course of fermentation compared to the other yeast microorganisms tested. Based on these data, we were also able to divide the strains into high fermentation strength strains (Y02 and Y05) and medium fermentation strength strains (Y01, Y03, Y06, and Y07).

Figure 3 shows the different fermentation progress of individual isolates. The consortium mixture was composed of isolates that showed different fermentation ability. The selection also included the incorporation of saccharomyces and non-saccharomyces yeast, which contribute to the sensory expression of the wine. Isolates Y03, Y06, and Y07 are characterized by a slower fermentation process. Isolates Y02 and Y05 were characterized by high sugar fermentability up to an alcohol content of 13.28 and 13.30% vol. alc., i.e. with a faster fermentation process. Y04 isolate showed the lowest sugar fermentability to an alcohol content of only 2.98, but the fermentation process was the most gradual.

H_2S production

Low, medium, and high sulfane productivity occurred during the monitoring of H_2S production by yeast isolates (Figure 4, Table 7). Most isolates (Y01, Y02, Y03, Y04, Y05) had medium sulfane productivity.

H_2S production was also monitored during the testing of a suitable fermentation strain, and strains with low, medium and high H_2S production were found. The Y07 strain showed low production; the Y01, Y02, Y03, Y04, and Y05 strains showed medium production; and the Y06 strain showed high production. Y02 and Y05 also showed high fermentation strength and medium H_2S production, while the Y07 strain produced a low amount of H_2S and had a medium fermentation capacity.

Discussion

This study focused on the isolation of yeasts and lactic acid bacteria representing the given vineyard. From the isolated microorganisms and growth curves, the most suitable culture media and required culture times were determined. The isolation was realized from the point of micro-sparkling of spontaneous fermentation due to the strain diversity. The study found that at an ethanol concentration stage of 4.5 – 5%, naturally, present non-saccharomyces yeasts die and ethanol-*tolerant S. cerevisiae* begins to act. Previous studies have also reported that many different strains occur at the beginning of fermentation, but only a few dominate in the later stages of wine fermentation **(Torija et al., 2001)**. Subsequently, fermentation tests were carried out to monitor the fermentation process and the fermentability of sugars to ethanol in individual isolates. Differences were found between individual isolates due to the isolation of microorganisms from the point of micro-sparkling when yeast diversity was greatest. During the fermentation tests, the differences in fermentability were shown, allowing us to select the yeast strain most suitable for fermentation.

Some similarities were found in the sugar fermentability of different isolates, specifically isolates Y02 and Y05 and Y01 and Y07. In the strains Y03 and Y06, there was always a certain deviation that distinguished them from the second group, and isolate Y04 showed a completely different course of fermentation compared to the other yeast microorganisms tested. Based on these data, we were also able to divide the strains according to strains with high fermentation strength (Y02 and Y05) and those with medium fermentation strength (Y01, Y03, Y06).

During spontaneous fermentation, different yeast species and strains interact with each other differently depending on the changing conditions of the fermenting must **(Albergaria and Arneborg, 2016; Ciani et al., 2016; Morrison-Whittle and Goddard, 2018)**. The medium becomes increasingly selective and this corresponds to the proportion of individual yeasts and bacteria **(Bisson, 2012; Perrone et al., 2013; Ciani et al., 2016; Brice et al., 2018; Henriques et al., 2018)**. Various studies indicate the prevalence of *S. cerevisiae* over non-saccharomyces, which usually initiate fermentation. *Saccharomyces* strains have greater tolerance to ethanol and temperature changes **(Goddard, 2008; Salvadó et al., 2011; Alonso-del-Real et al., 2017)**.

Ganucci et al. (2018) reported the effect of ethanol and temperature on the dominance of various *S. cerevisiae* strains occurring in multiple spontaneous fermentations carried out on an industrial scale. Another study by **Tofalo et al. (2013)** examined the prevalent strains of *S. cerevisiae*, which were differentiated by the RFLP-mtDNA method and according to their isolation frequency. The results obtained by an analysis of 637 isolates

confirmed the genetic polymorphism expected in the *S. cerevisiae* population in spontaneous wine fermentation and the high variability between isolation frequencies of different strains. **Schuller et al (2012)** evaluated intraspecific genetic diversity of fermentative vineyard-associated *S. cerevisiae* strains and evaluate relationships between grape varieties and geographical location on populational structures. Similar results are shown in the study **(Bisson, 2012; Schuller et al., 2012; Tofalo et al., 2013)**.

The study by **Ganucci et al. (2018)** further found that independent of the grape variety, five of the six wineries in the study only had one predominant *S. cerevisiae* strain with an isolation frequency ranging from 32 to 74%, while the variable number of strains (from four to 14) was characterized by an isolation frequency of less than 10%. This finding is consistent with those reported by other authors **(Versavaud et al., 1995; Gutiérrez et al., 1997; Egli et al., 1998; Sabate et al., 1998)**, although in some cases the predominant strains of *S. cerevisiae* were not found by the fermentation process **(Vezinhet et al., 1992)**.

To select the most suitable yeast strain for fermentation, H_2S production was also monitored on a special nutrient medium: BIGGY. The obtained isolates showed varying degrees of H_2S production, ranging from low, medium to high H_2S production. These results confirm those presented by **Perrone et al. (2013)** and **Pérez-Torrado et al. (2017)**, which state, inter alia, that the dominant behavior of yeast strains is due to differential H_2S production and killer factor resistance.

It is noteworthy that in the high-frequency strains that were tested by **Ganucci, Guerrini et al. (2018)**, no killer factor was detected and no significant differences in H_2S production were found. The degree of competition of each strain, which determines the ability of one strain to compete with another, is influenced by other factors, however, including pH, temperature, ethanol, osmotic pressure, and available nitrogen **(Ciani et al., 2016)**.

Ganucci et al. (2018) study looking at the effect of ethanol and temperature on growth performance and condition advantage of high-frequency *S. cerevisiae* strains showed that these two factors can play an important role in determining the dominance of one strain over another during wine fermentation. A single action of ethanol on the growth performance led to the high-frequency strains showing significantly lower inhibition than the low-frequency strains.

According to **Arroyo-López, Querol and Barrio (2009)**, an even more accurate indicator of total yeast growth is the percentage of inhibition as this parameter is indirectly related to the delayed phase but linearly related to both the maximum specific growth rate (μmax) and the maximum cell density at the end of growth. Consequently, there is an advantage of condition, which according to **Salvadó et al. (2011)**, represents the difference in μmax between competitors for specific environmental conditions. This leads to higher concentrations of high-frequency strains, indicating their enhanced adaptability to increasing ethanol concentrations compared to low-frequency strains. Each *S. cerevisiae* strain can exhibit different stress reactions to ethanol because the effects of increasing ethanol concentrations on the yeast cell include various changes, such as membrane composition and gene expression, synthesis of heat shock proteins, increase in chaperone proteins, etc.**(Ding et al., 2009)**.

Another study of four commercial wine yeast strains recently highlighted that fermentation temperature may be an important factor in determining the dynamics of a population of *S. cerevisiae* strains **(García-Ríos et al., 2014)**. Ethanol and high temperature synergistically affect membrane integrity and permeability, causing a decrease in yeast population growth **(Alexandre, Rousseaux and Charpentier, 1994; Albergaria and Arneborg, 2016)**.

CONCLUSION

The outcome of this study was the 2018 Wine Microorganism Consortium, which was obtained from the spontaneous fermentation that characterizes the given vineyard and supports the 'terroir' of the wine. At the same time, inoculation with this mixed culture helps to prevent problems with stagnant fermentation, which is often associated with spontaneous fermentation. Isolates of lactic acid bacteria and non-saccharomyces and saccharomyces yeasts were obtained and the resulting consortium was formed from these isolates. The obtained consortium was then used for fermentation tests where the percentage of fermented sugar and hydrogen sulphide production were monitored.

The Y03, Y06, and Y07 isolates had the largest mass representation and were characterized by a looser fermentation, but with the ability to ferment alcohol to 6.98, 9.37, and 7.20 vol. % alc. These isolates showed low, medium, and high ability to produce H2S. Y02 and Y05 isolates had the second largest mass representation of the resulting consortium and were characterized by high sugar fermentability up to an alcohol content of 13.28 and 13.30% vol. alc., but the fermentation process was not gradual, which could negatively affect the quality of the wine. Both isolates showed medium ability to produce H2S. Y04 isolate also has a low mass representation and showed the lowest ability to ferment sugar to an alcohol content of only 2.98 and a medium ability to produce H2S.

The Y03, Y06, and Y07 isolates had the largest percentage in the resulting consortium. These isolates formed 80% of the total weight of the consortium. The LAB 01 and LAB 02 isolates represented 7% of the consortium. Lactic acid isolates do not participate in alcoholic fermentation and are in the consortium to start malolactic fermentation.

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ASSESSMENT OF THE INTAKE OF SELECTED MINERALS IN POPULATION OF PREMENOPAUSAL WOMEN BASED ON SPECIFIC SOCIO-DEMOGRAPHIC INDICATORS

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ABSTRACT

Eating behavior interventions are a modifiable risk factor for chronic diseases. The aim of this study was to monitor the intake of selected minerals – calcium, phosphorus, zinc, copper, selenium, and chromium in the diet of premenopausal women (n = 30) and to highlight the possible adverse effects of disbalanced intake of these elements. At the same time, we investigated whether socio-demographic factors and choice of food store have an impact on the intake of these substances. We assessed the intake of selected minerals using three-day nutritional protocols and subsequently processed the data. The participants were women between 40 and 50 years old, from districts of Partizánske and Prievidza, for whom menopause has not yet begun. Women provided information about their place of residence (urban or rural area), type of home they live in (family house or apartment), and the type of food store where they grocery shop. The results indicate an impact of the place of residence: higher intake of zinc ($p = 0.012$) and selenium ($p = 0.020$) were observed in the participants from the urban area. The impact of the type of home was proven on the intake of chromium ($p = 0.049$), copper ($p = 0.048$), and carbohydrates ($p = 0.021$) with higher intake in the apartment-dwelling group. The impact of food store choice has not been confirmed. Based on the observed values, we conclude that the observed population might be at a higher risk of skeletal disorders and osteoporosis due to deficient calcium intake and the unfavorable ratio of Ca:P; increased Zn and Se intake levels may stimulate the development of cardiovascular risk factors and may also elevate the risk for type 2 diabetes mellitus.

Keywords: minerals; intake; diet; menopause; place of residence; type of home; type of food store

INTRODUCTION

The basic need of the human body is food intake. Food supplies building material and energy and contains immune-system-promoting substances. Macronutrients (proteins, fats, and carbohydrates) and micronutrients (minerals and vitamins) are introduced into the body through food **(Svačina et al., 2008)**. Macronutrients supply energy and material to ensure the entire body composition. Micronutrients are required to maintain continuous design and reconstruction processes. The micronutrient requirements of an organism vary depending on individual needs **(Biesalski and Tinz, 2018)**. The primary functions of micronutrients in human metabolism and physiology are to maintain and optimize health and prevent disease. Adequate intake is essential to maintain homeostasis, physiological functioning, and normal growth and development of a young organism **(Shergill-Bonner, 2017)**. All vitamins and minerals can be obtained from a balanced diet that includes all food groups from the food pyramid. It has been known since the 18th century that diseases may result from low-quality food **(Combet and Buckton, 2019)**. Micronutrients are essential dietary ingredients with preventive effects. **Shergill-Bonner (2017)** states that chemical reactions in different metabolic pathways may not be able to continue their natural pathway if a critical micronutrient is missing. Normal metabolic regulation of the organism will be impaired and poor health can develop due to the lack of specific micronutrients. The physiological functions of micronutrients include acting as coenzymes in key metabolic reactions, antioxidants to control damage caused by reactive oxygen species, gene transcription modulators, enzyme components and cofactors, and structural tissue components **(Combet and Buckton, 2019)**.

From the publication "Slovakia: country health profile 2019" we learn that the most frequent cause of death of women in Slovakia is cardiovascular diseases (50% of deaths) and cancer (24%) **(OECD, 2017)**. During menopause, the female body undergoes changes that gradually increase the risk of developing diseases. The production of endogenous estrogens with anti-atherosclerotic and anti-inflammatory properties is decreasing, maintaining pancreatic insulin response to glucose **(Svatikova and Hayes, 2018; Wedisinghe and**

Perera, 2009). According to **Harvey, Coffman and Miller (2015)**, loss of estrogen contributes to the increased development of hypertension, ischemic heart disease, congestive heart failure, and cerebrovascular disease. Of the micronutrients, selenium, zinc, and copper are particularly critical in preventing cardiovascular disease (CVD). Severe selenium deficiency is a known cause of reversible heart failure – Keshan disease. In patients with CVD, a disbalance is often observed – increased copper levels and concurrently reduced levels of zinc and selenium in the body **(Koşar et al., 2006; McKeag et al., 2012; Salehifar et al. 2008)**. Calcium is also important in the prevention of chronic diseases; it is a modifiable risk factor for osteoporosis **(Skowrońska-Jóźwiak et al., 2016)**, reduces the risk of hypertension and colon cancer **(Ong et al., 2017)**, and normalizes blood levels. Its low intake is associated with pathogenesis of obesity, hypertension, insulin resistance, and type 2 diabetes **(Skowrońska-Jóźwiak et al., 2017)**. In addition to Ca, phosphorus is also essential for bone tissue – together they form hydroxyapatite **(Itkonen et al., 2017)**. Furthermore, plasma levels of P inversely correlate with body weight **(Zohal et al., 2019)**.

Zinc is a trace element that plays a role in over 300 biological processes. It plays an important metabolic role in the metabolism of proteins, carbohydrates, lipids, and nucleic acids. It affects the action of insulin and is an integral part of many antioxidant enzymes. Its deficiency damages the synthesis of these enzymes, which increases oxidative stress **(Zohal et al., 2019)**. A high incidence of Zn in the brain (amygdala, hippocampus, neocortex) has been observed and found to have several important effects on the CNS. It is believed that inadequate Zn intake may be associated with various changes in mental functions (e.g. behavior, cognition, and mood) **(Dome et al., 2019)**.

Copper is a cofactor of redox enzymes (ceruloplasmin in iron metabolism), participates in antioxidant defense, neuropeptic synthesis, and immune responses, and it is also important in wound healing and haematopoiesis **(Bost et al., 2016; Uzzan et al., 2017)**. Higher copper intake may increase the risk of stroke, other cardiovascular diseases, and overall CVD mortality. The exact mechanism is unknown, but it is believed that copper can be incorporated into the molecule instead of zinc or other metals during protein biosynthesis, also oxidizing LDL-cholesterol, thereby increasing its atherogenicity. Copper can also be considered a risk marker of inflammation through its relationship with the acute phase reactant, ceruloplasmin. Cu overload is associated with insulin resistance: high serum copper levels have been found in patients with type 2 diabetes **(Eshak et al., 2018)**.

Current data support the beneficial effects of selenium on hypertension, coronary artery disease, cancer, and inflammatory diseases **(Asemi et al., 2015)**. Studies have shown a significant decrease in serum insulin levels and a reduction in insulin resistance in obese women after supplementation with 200 $mg.day^{-1}$ Se, added to a hypocaloric diet enriched by legumes **(Alizadeh et al., 2012)**. Se deficiency is a factor in the development of cardiovascular and neurodegenerative diseases, aging, and immune system damage due to oxidative stress **(Wang et al., 2014)**.

Chromium (Cr^{3+}) supplementation improves insulin sensitivity and blood glucose levels in animals and humans with impaired glucose tolerance, insulin resistance, and diabetes **(Staniek and Wójciak, 2018)**. In contrast, Cr^{6+} is very toxic and has a high ability to enter cells, causing a wide range of damage – DNA damage, chromosome aberrations, changes in epigenome, and microsatellite instability **(Bjørklund et al., 2017)**. It causes various types of cancer; exposure to Cr^{6+} can result in asthma and damage to the nasal epithelium and skin, and the effect of Cr^{6+} on the thioredoxin system likely has widespread consequences for cell survival and redox signaling in cells **(Kaprara et al., 2015)**.

Scientific hypothesis

The female organism undergoes changes during menopause resulting from a gradual decrease in estrogen production that increases several health risks. Considering these risks, we selected 6 micronutrients, the intake of which we will evaluate. We assume that women living in a city environment, mostly in apartments, will show a higher intake of micronutrients. We also examine whether the choice of shopping place influences the intake of selected minerals or energy intake.

MATERIAL AND METHODOLOGY

We monitored the intake of selected minerals in the diet of premenopausal women and highlighted the possible adverse effects of disbalanced intake of these elements. At the same time, we investigated whether socio-demographic factors and choice of food store have an impact on the intake of the mineral substances. 30 women aged between 40 and 50 years old (44.79 ±2.04 years), from the districts of Partizánske and Prievidza, were involved in the research. Eating habits and intake of monitored nutrients were determined using 3-day dietary records, which included 2 workdays and 1 non-workday. All participants reported a weekend day, mostly Sunday (23 of 30). We collected the nutritional protocols from October 2018 to January 2019. For BMI determination self-reported weight and height were used. All participants participated in the research voluntarily, were acquainted with the way of processing the provided data, and provided their consent to their processing.

The assessed nutritional parameters included the intake of energy and essential macronutrients – carbohydrates, protein and fat, and intake of selected micronutrients – calcium, phosphorus, zinc, copper, selenium, and chromium, which affect the health of the participants in relation to certain health risks arising from the upcoming menopause. The study was aimed at nutritional intake, therefore the level of physical activity was not a necessary criterion.

We evaluated the data using Mountberry nutritional and fitness software (2011, version 1.1). This software is designed for a complete analysis of food, meals, and recipes based on the composition of the raw ingredients. Using the updated food database, it is possible to adjust the software outputs in terms of nutrient intake, health ailments, dietetic principles, and individual user needs. Dietary regime analysis evaluates energy and nutrient balance and the intake of selected nutrients and compares

the values with the recommended standard. If the food listed in the nutritional protocol was not contained in the software database, we added the nutritional data manually into the database. We also proceeded in cases, in which, in order to add the food into the database, we had to consult with the participant about the exact recipe.

Statistical analysis

Statistical analysis was carried out using MS Excel 2010 (Los Angeles, CA, USA) in combination with XLSTAT (Version 2019.3.1). Mean, standard deviation, minimum and maximum, and median were calculated. Statistical significance was determined using a two-sample t-test when comparing subgroups of the population and a paired t-test when comparing the nutritional intake of the entire population during a workday and weekend. Differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Workday vs. weekend

Significant adverse effects on the intake of basic nutrients and energy were observed on workdays and non-workdays, for which all participants provided a weekend day (Table 1).

On weekdays, women tend to receive lower amounts of total energy, protein, carbohydrates, and fats compared to a weekend day. According to the calculated mean values, the recommended daily allowance (RDA) (57 g) was met only for protein intake, both on weekdays (62.89 ±27.33 g) and over the weekend (75.47 ±25.76 g).

We observed that the average values of the monitored group did not meet the RDA guideline **(MHSR, 2015)**, which are 8800 kJ of energy, 306 g of carbohydrates, and 72 g of fats. At the same time, there are obvious differences in the intake of the selected minerals on a workday and weekend day.

Haines et al. (2003) described high energy intake from alcoholic beverages over the weekend, especially on Saturday, in the 19 – 50 age group, as did **An (2016)** and **Jahns et al. (2017)**. In addition to alcohol, energy intake was also increased by the increase in the proportion of fats in the weekend diet, which was richer than during workdays by an average of 115 kcal (481.28 kJ).

Haines et al. (2003) observed similar results as the study of **An (2016)**, which examined the differences in the nutrition of 11 646 adults over 18 years in the United States during workdays and weekends. In comparison with the average values on workdays and on Saturday, the weekend intake was increased, especially in the case of total energy, by 181.04 kcal, approximately 757.5 kJ. Food quality on Saturday was lower than on other monitored days.

Jahns et al. (2017) also found a higher energy intake of macronutrients over the weekend in a population of middle-aged women (49.4 ±5.8 years). In 75% of these women, they noticed increased energy intake by an average of 158 kcal (661.23 kJ) over the weekend. The increase in intake was provided mainly by carbohydrates, as in our results. The least variable nutrient were fats; their intake was very similar during the week, contrary to our results. In our research, we observed very small differences in protein intake.

From our results, on workdays, the recommended nutritional quantities of protein intake were met only in 56.67% of cases, and those of fat intake in 6.67% of cases in a population of thirty. None of the participants from the observed group met the recommended intake of energy and carbohydrates. Weekend intake was higher for all four endpoints (Tables 1), with recommended energy intake met by 23.33%, protein intake by 73.33%, carbohydrates intake by 16.67%, and fat intake by 40.0% of the participants.

Table 1 Average intake of monitored nutrients, working day vs. weekend day.

Parameter	Work day		Weekend day		*p*-value[1]
	Mean ± SD	median	Mean ± SD	median	
Energy (kJ)	4989.75 ±1283.91	4906.86	7440.07 ±2504.7	7101.47	0.000001[2]
Protein (g)	62.89 ±27.33	59.73	75.47 ±25.76	66.83	0.011115[2]
Carbohydrates (g)	153.83 ±39.86	151.11	231.68 ±74.81	217.22	0.000001[2]
Fats (g)	44.5 ±16.49	44.23	68.47 ±28.32	64.26	0.000074[2]
Ca (mg)	613.62 ±241.37	564.89	657.28 ±325.62	560.64	0.25
P (mg)	913.71 ±282.62	824.05	1024.28 ±290.86	999.69	0.07
Zn (mg)	25.85 ±12.46	23.63	25.99 ±14.02	22.70	0.47
Cu (mg)	1.39 ±0.82	1.16	1.31 ±0.46	1.18	0.28
Se (µg)	177.13 ±106.46	180.34	163.37 ±102.15	165.04	0.15
Cr (µg)	38.00 ±12.57	37.62	41.88 ±26.42	37.85	0.25

Note: [1] statistically significant differences were verified using paired t-test; [2] $p < 0.05$ was considered statistically significant.

Table 2 Average values of monitored parameters, living in urban vs. rural area.

Parameter	Urban area (n = 17)		Rural area (n = 13)		*p*-value[1]
	Mean ± SD	median	Mean ± SD	median	
	23.76 ±4.27	22.68	23.32 ±3.16	23.38	0.38
Energy (kJ)	5733.78 ±1531.09	5540.84	5901.62 ±1317.57	5833.61	0.38
Protein (g)	62.61 ±19.16	64.06	73.40 ±27.28	64.51	0.13
Carbohydrates (g)	177.02 ±45.01	179.20	182.92 ±40.99	168.92	0.36
Fats (g)	51.96 ±18.00	54.42	56.86 ±22.91	49.04	0.27
Ca (mg)	617.49 ±289.56	616.43	642.14 ±323.23	572.48	0.39
P (mg)	944.25 ±351.47	920.38	959.02 ±312.67	991.80	0.43
Zn (mg)	30.05 ±14.86	32.47	20.47 ±11.14	20.50	0.012[2]
Cu (mg)	1.47 ±1.22	1.36	1.22 ±0.42	1.15	0.13
Se (µg)	204.02 ±118.58	227.67	131.39 ±101.80	129.17	0.020[2]
Cr (µg)	41.98 ±21.28	45.05	35.77 ±20.89	35.92	0.08

Note: [1] statistically significant differences were verified using two-sample t-test; [2] $p < 0.05$ was considered statistically significant.

Table 3 Average values of monitored parameters, living in family house vs. apartment.

Parameter	Family house (n = 16)		Apartment (n = 14)		*p*-value[1]
	Mean ± SD	median	Mean ± SD	median	
BMI (kg.m^{-2})	22.93 ±4.51	22.06	24.29 ±2.70	24.04	0.17
Energy (kJ)	5436.55 ±1307.28	5365.77	6286.70 ±1477.32	5795.05	0.06
Protein (g)	67.66 ±27.80	63.27	68.51 ±18.19	67.77	0.46
Carbohydrates (g)	165.12 ±40.09	164.92	197.63 ±39.97	191.31	0.0
Fats (g)	53.08 ±21.96	51.14	55.29 ±18.47	54.48	0.39
Ca (mg)	580.76 ±183.66	528.15	682.36 ±243.77	639.05	0.12
P (mg)	916.45 ±241.29	797.61	989.73 ±185.83	969.20	0.19
Zn (mg)	24.40 ±12.52	21.34	27.60 ±11.57	24.25	0.24
Cu (mg)	1.16 ±0.28	1.10	1.59 ±0.83	1.32	0.048[2]
Se (µg)	157.58 ±106.25	151.73	189.65 ±88.90	169.41	0.20
Cr (µg)	36.00 ±11.15	36.77	43.05 ±10.64	45.28	0.049[2]

Note: [1] statistically significant differences were verified using two-sample t-test; [2] $p < 0.05$ was considered statistically significant.

Several authors **(Haines et al., 2003; Ruopeng, 2016; Jahns et al., 2017)** have observed lower nutritional quality of the diet over weekends – reduced intake of fruits, vegetables, and fiber, and increased consumption of fast food. Our results, on the other hand, show better nutritional status over the weekend, especially in terms of total energy, carbohydrate, and fat intake, but participants also received higher amounts of almost all minerals (except Cu and Se). Based on the average intake of the selected nutrients, we find that the intake of micronutrients over the weekend is almost the same as on weekdays. The biggest differences were in the intake of energy, carbohydrates, and fats, which is explained by the fact that during the weekend, the participants had time to consume more meals per day compared to a workday (average of 4.5 meals on a weekend day; an average of 4.1 on a workday). Of those involved, 53% received at least one meal more during the weekend than on weekdays.

Evaluation of the intake of monitored nutrients by place of residence (urban vs. rural area)

Urbanization interacts with several key determinants of food consumption. It is believed that the self-production of food is not very common in urbanized areas, which also affects dietary habits **(Cockx, Colen and De Weerdt, 2018)**.

From the monitored population of 30 women, 13 women lived in a village and the remaining 17 lived in a city. Based on average values of the group (Table 2), intake of minerals, zinc, copper, selenium, and chromium were met in both groups of women, even exceeding the

recommended amounts given in the RDA guideline **(MHSR, 2015)**.

In the "urban" group, we observed that the average BMI was higher than in the "rural" group, mainly because most overweight (>25 kg.m^{-2}) and obese (>30 kg.m^{-2}) women lived in the city (Table 3). Although BMI above 30 kg.m^{-2} was observed only in 2 participants in our study, several studies of women of similar age categories show the prevalence of higher BMI in urban women, often in association with waist circumference (≥88 cm) **(Okop, Levitt and Puoane, 2015; Rothman et al., 2018)**. In addition, **Okop, Levitt and Puoane, (2015)** reported a higher proportion of excessive body fat in urban women (47.6 ±11% body fat) compared with women living in rural areas (44.10 ±10% body fat), differences in BMI parameters (34.7 ±9 and 31.4 ±8 for city and village, respectively), and waist circumference (100.1 ±16 cm in the city and 93.7 ±17 cm in the village). The percentage of women in the 35 – 49 age group (n = 429) with high measured endpoints was 10% higher for BMI, 6% higher for waist circumference, and 4% higher in the urban group compared to the rural. Conversely, **Trivedi et al. (2015)** are critical of the rural group in the American population, in which they observed a higher prevalence of obesity due to poor eating habits and lower physical activity. The prevalence of female obesity was 33.4% in the rural area and 28.2% in the urban area (p <0.01). The nutritional risk factors associated with an increased risk of obesity were lower fruit consumption, higher protein intake (mainly from meat and beans), and skipping breakfast.

Both groups met the requirements for the recommended intake of phosphorus ingested – the intake exceeded the RDA by more than 200 mg. However, in the case of calcium, the intake was almost 400 mg below the RDA in both groups. Women living in the village environment received a higher amount of Ca, which is surprising given that the intake of other minerals was lower than in the urban group. However, on average, this amount was still insufficient, and less than 30 mg higher than that of the women from the city district. **Rothman et al. (2018)**, in their study of 452 women (aged 45 to 54 years), observed a higher intake of milk and dairy products (Ca sources) in the diet of the urban population compared to the rural population and assumed that calcium intake is directly proportional to the consumption of these foods. They refer to an insufficient intake of these foods and thus calcium as a risk factor in the development and progression of osteoporosis.

Average zinc intake was elevated almost 2.5 to 3 times the recommended daily dosage, which may be counterproductive and may disrupt the homeostasis of the body. However, the mean value was not within the range of 50 – 300 mg.d^{-1}, and therefore we assume that chronic toxicity manifestations of this element should not be present **(EFSA, 2006)**. The dietary intake of copper according to the RDA guideline **(MHSR, 2015)** is 900 µg and 1.6 mg according to the Mountberry nutritional software used; therefore, the values obtained from the nutritional records of the population were optimal. Again, we observed a higher intake of both elements in the group of participants living in the city.

Ilow et al. (2011) also reported lower zinc intake in urban areas, although our average values were 3 to 4 times higher than those reported, mainly attributed to the size of our sample (n = 30) and the sample size of **Ilow et al. (2011)** (n = 2572). However, copper intake in their study was higher in rural areas by an average of 0.1 mg, while we observe a higher intake of 0.25 mg in the urban population. In both cases, copper intake exceeds the recommended daily quantities.

A phenomenon similar to that of zinc intake is also observed for selenium intake. The urban part of the population receives a higher amount than women living in the village, and the intake is 2 – 3 times higher than recommended by the Ministry of Health. If the participants consistently received that much selenium, as in the case of zinc, their organisms could be damaged. Chromium intake only slightly exceeds the recommended intake in women living in a village and can be said to be optimal compared to Cr intake in the city where we observe a slightly higher average intake value. Nutritional intake is again higher in the urban group.

Table 4 Average values of nutrition parameters, supermarket vs. different food source.

Parameter	Different food source (n = 18)		Supermarket (n = 12)		p-value[1]
	Mean ± SD	median	Mean ± SD	median	
BMI (kg.m^{-2})	24.1 ±4.45	22.46	22.77 ±2.45	22.51	0.16
Energy (kJ)	5765.41 ±1408.54	5718.56	5868.17 ±1495.54	5597.32	0.43
Protein (g)	63.30 ±18.47	62.29	73.27 ±28.75	66.68	0.16
Carbohydrates (g)	180.94 ±43.41	170.13	177.54 ±43.34	184.97	0.42
Fats (g)	56.28 ±22.63	54.04	50.78 ±15.99	51.73	0.23
Ca (mg)	644.03 ±237.46	555.72	604.39 ±187.61	595.82	0.31
P (mg)	923.76 ±212.40	916.74	990.99 ±225.56	933.49	0.22
Zn (mg)	23.60 ±11.34	21.99	29.34 ±12.60	26.39	0.12
Cu (mg)	1.21 ±0.28	1.17	1.58 ±0.91	1.27	0.11
Se (µg)	156.91 ±105.50	137.68	196.00 ±85.44	182.83	0.15
Cr (µg)	36.69 ±11.81	38.13	43.20 ±9.73	45.28	0.06

Note: [1] statistically significant differences were verified using two-sample t-test; [2] p <0.05 was considered statistically significant.

Similar findings were present in the Polish population, with higher Se intake in cities (77.3 ±31.3 μg per day) compared with rural areas (72.9 ±23.6 μg per day). The authors attributed the size of the standard deviation to different numbers of participants (n = 1786 for the urban population and n = 786 for the rural population) **(Ilow et al., 2011)**.

The differences in the diets can be explained by the fact that urban areas are characterized by a significantly different food supply environment, affecting the availability and price of food. The possibilities of eating away from home or buying semi-finished or ready-made meals are more prevalent and more diverse in urban areas because there are mini-markets, supermarkets, and fast-food chains with easy availability of food **(Cockx. Colen and De Weerdt, 2018)**.

Evaluation of the intake of monitored nutrients by type of home (family house vs. apartment)

The participant group comprised of 16 women living in a family house with a garden and 14 living in an apartment. The average intake of minerals was similar to the previous criterion of evaluation, namely that the intake of all selected micronutrients was sufficient except for calcium, intake of which was deficient in both groups regardless of the type of housing (Table 3).

We observed a higher intake of Ca in participants living in a family house with a difference of over 100 mg. Six women from this group achieved the recommended intake, but none received more than 1000 mg twice or more in 3 monitored days. In the group of women living in apartments, we have seen such values only in 4 women, also only one day from the three-day nutritional protocol. Phosphorus intake was met according to RDA limits but may be of concern due to low calcium intake. The calcium to phosphorus intake ratio in the "family house" group was 1:1.58, while in the "apartment" group the Ca:P ratio was 1:1.45. **EFSA (2015)** reports an optimal Ca:P ratio of 1.4:1 to 1.9:1 and may therefore be a risk factor at a later age or after menopause, particularly in relation to osteoporosis.

The mean value of Zn intake was 27.60 ±11.57 mg in the group of women living in apartments and 24.40 ±12.52 mg in family houses. The actual intake was more than triple the RDA, but this amount should not cause any health risks according to **EFSA (2006)**.

Comparing the intake of copper in both groups, we can see that the individual average received quantities are higher than those stated by the **MHSR (2015)** in the RDA for SR, therefore, the intake was sufficient. However, according to the limits in Mountberry software, which recommends an intake of 1.6 mg Cu per day for the selected age group, intake would be inadequate, especially in the "family house" group.

The average Se intake in the "apartment" group was more than three times the RDA. For women living in a family home, we also see a higher daily intake, but to a lesser extent. Selenium intake is high throughout the population regardless of assessment criteria, which may have an adverse effect on the cardiovascular system, as reported by **Grotto et al. (2018)** and **Vinceti et al. (2019)**. **Grotto et al. (2018)** reported that high intake induces changes in blood pressure: an increase in systolic blood pressure. Their experiments were performed in mice which they had been feeding increased amounts of selenium for 85 days via drinking water (2 and 6 $mg.L^{-1}$) in the form of sodium selenite; the first changes in blood pressure were observed after 42 days. Although several authors report 400 μg a day as a critical intake of Se in humans, and according to the recorded average values, the respondents' health should not be in danger, we do not recommend a multiple increase in the intake of this mineral.

According to the RDA, the intake of chromium is adequate in both groups, with a more optimal value in the "family house" group, where the difference from RDA is on average 1.01 μg, while in women living in apartments, the actual intake exceeds RDA by less than 23%. Overall, we note that the intake of Cr and Cu shows the most optimal values in relation to the recommended daily allowances set by the Ministry of Health of the Slovak Republic **(MHSR, 2015)**.

We can see from Table 3 that the intake of all nutritional parameters was higher in women living in apartments. **Cockx. Colen and De Weerdt (2018)** suggest that smaller living space and a lack of storage and cooking facilities could contribute to increased dependence on foods that require less or no preparation. This phenomenon may encourage more frequent shopping and more varied food choices, depending on the individual's current preferences.

Evaluation of the intake of monitored nutrients by food source (supermarket vs. mixed source)

Several studies point out that the choice of grocery store affects food intake and nutritional composition. We anticipated that total energy intake would be higher in the supermarket group, due to the increased availability of semi-prepared foods and ready-made meals that tend to contain more salt, saturated and trans-fatty acids, and sugar **(Albuquerque et al., 2018)**.

Most women (18) reported different sources of food than the supermarket, which included local producers, vendors, farmers, or their food production. The second group consisted of women (12) who identified supermarkets as their only food source – they did not buy food in other establishments.

We observed that the intake of minerals, except Ca, is higher in the group buying food in several stores of local farmers and producers or growing and producing the food themselves (Table 4). In this group we also see a better ratio of calcium to phosphorus intake, numbering 1:1.43 compared with 1:1.64 in the supermarket only group. From this point of view, women buying only in supermarkets could be at a higher risk of breaching bone homeostasis. Regardless of the Ca: P ratio, calcium is deficient in the diet of the participants of both groups. This is not true of phosphorus intake, which seems excessive compared to RDA in both groups and reaches 141.57% of RDA in the supermarket group and 131.97% RDA in the "different food source" group.

Zinc intake is excessive in both subgroups but is significantly higher in women shopping at the supermarket. Zinc, due to its role in the human body, is nowadays one of the elements by which food, for example, breakfast cereals or flour, is fortified, especially to prevent its deficiency in the body **(Brown, Hambidge and Ranum, 2010; Shah et al., 2016)**. The likelihood of availability of such enriched foods in local farm shops is

lower than in the supermarkets. Again, the average amounts received are within the range recommended in the RDA and Mountberry (0.9 – 1.6 mg per day), leading us to conclude that the diet intake is optimal.

The amounts of selenium and chromium received from the diet were met in terms of the recommended daily intakes in both groups. Again, as with most previous micronutrients, higher quantities were received by women shopping in the supermarket.

In our representative sample of women, it was not confirmed that the choice of food source had an impact on the intake of the micronutrients monitored, as the differences between the groups are slight and statistically inconclusive (Table 4). These findings are consistent with **Cummins, Flint and Matthews (2014)**, who reported that they did not find significant changes in endpoints when changing the typical grocery shopping place in the American population. However, **Liese et al. (2017)** noted the relationship between BMI and the primary grocery store. Their research involved 459 respondents (80% women), with 61% listing a supermarket as their primary point of purchase. Their results pointed to an interesting association: higher BMI values of 2.6 $kg.m^{-2}$ were found in people who regularly and primarily shopped in large stores (supermarkets) and discount halls compared to shoppers in small local operations.

In the group of women shopping in supermarkets, we observed a slightly higher energy intake compared to the second group, but the nutrient intake did not change significantly, which may mean that these participants received more low-nutrient but high-energy foods. This, according to **Cohen et al. (2015)**, can reflect in changes in body weight and consequently an increase in BMI. More frequent consumption of such foods is also helped by the marketing strategies of the producer or retailer: lower price, bigger packaging, and advantageous offers (2 in 1). Based on the results (Table 4) we can say that the choice of food source does not play a significant role in the intake of minerals. We observe minor abnormalities in the intake of macronutrients, but given the uneven distribution of the population with a higher number of participants on the mixed food source side (n = 18) than on the supermarket side only (n = 12), we conclude that these differences are due only to the food preferences of the women.

Several strengths and limitations exist in the current study. We focused strictly on the intake of selected nutrients and did not consider physical activity, which may increase the body's need for some nutrients. Another limitation might be the sample size. On the other hand, the strength of the study is the age group and the quality of the obtained nutritional data. Collected 3-day dietary records were responsibly filled by participants and we completed the dietary records with an interview about used groceries and food processing.

CONCLUSION

We assessed the impact of a diet in premenopausal women in relation to the risk factors for chronic non-communicable diseases, the development of which is related to the menopausal period.

Mineral intake was adequate with average values higher than the RDA guideline by MHSR, except for calcium. Based on the place of residence, we observed a higher intake of macronutrients and macroelements (Ca and P) in participants living in the village, but the intake of other minerals was lower compared to the urban group. There were statistically significant differences in the intake of Zn ($p = 0.012$) and Se ($p = 0.020$), but the place of residence did not affect the intake of other nutrients. The effect of the type of home was statistically significant only in the case of intake of Cr ($p = 0.049$) and Cu ($p = 0.048$) with higher intake in participants living in an apartment. An increase was also observed in carbohydrate intake ($p = 0.021$). In the case of shopping place location, there was no statistically proven effect on the intake of minerals or macronutrients.

Based on the current intake of the monitored nutrients and assessment criteria, we conclude that the study group is at risk for the development of osteoporosis due to overall insufficient Ca intake in the entire population sample. We also take into account the risk of developing type 2 diabetes mellitus due to abnormal Se and Zn intakes and an increased risk of cardiovascular complications that may facilitate the development of metabolic syndrome in the future, especially in women living in the city.

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PREPARATION OF PROTEIN PRODUCTS FROM COLLAGEN-RICH POULTRY TISSUES

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ABSTRACT

Chicken stomachs are by-products obtained from the poultry processing in slaughterhouses. Their amount has been gradually increasing as a consequence of a continually rising poultry consumption. Since these animal tissues are still rich in proteins, mainly collagen, fat, and minerals, it is essential and beneficial to investigate the appropriate management and further processing. Collagen could be extracted from chicken stomachs and used as a raw material in the food, cosmetic, medical, and also pharmaceutical industry. This paper is to investigate possibilities of such extraction of collagen products, gelatines, or alternatively hydrolysates, from chicken stomachs after prior biotechnological treatment with the proteolytic enzyme Protamex. In this experiment, non-collagenous proteins were removed from stomachs using 0.03 M NaOH and 0.2 M NaCl. Subsequently, the tissue was defatted applying acetone and the enzyme Lipolase. Purified and dried collagen was then treated with the proteolytic enzyme Protamex. In the last step, gelatine was extracted from the tissue in hot water. The influence of selected processing parameters on the extraction efficiency and final product quality was monitored. The extraction conditions included the amount of the added enzyme (0.1 – 0.4%) and the extraction temperature of between 60 and 65 °C. The total gelatine yield ranged from 43.80 to 96.45% and the gel strength varied from 2 ±0 to 429 ±8 Bloom. The enzymatic treatment of the raw material is an economical and ecological alternative to traditional acid or alkaline treatments. Extracted gelatine with the gel strength of 100 – 300 Bloom would be suitable for the applications in the food industry in the production of confectionery, marshmallow, aspic or dairy products.

Keywords: biotechnology; chicken stomach; food industry; by-products; gelatine

INTRODUCTION

The consumption of poultry meat has been consistently increasing. The current status in the Czech Republic is approximately 27 kg per person per year. Such a situation emphasizes the importance of management and processing of slaughter by-products **(Český statistický úřad, 2019)**. The poultry slaughter process produces two forms of edible and inedible waste, solid and liquid. Solid waste includes skin, feathers, intestines, offal, glands, limbs, and bones and liquid involves blood and various adipose tissues **(Seong et al., 2015; Ockerman and Hansen, 2000)**. Poultry waste comprises up to 30% (in several cases even 40%) of the live weight of the animal. Considering their proteinaceous nature and the fact they are produced in large quantities, they must be managed to avoid environmental pollution. However, inedible parts of poultry are mostly incinerated or landfilled. That is undesirable waste management producing up to 100 million tonnes of waste worldwide **(Borowski and Kubacki, 2015; Xiong et al., 2016; Ferraro, Anton and Santé-Lhoutellier, 2016)**. Blood is used as an additive in certain food products and in the production of feed meal. Bones and skins are applied in the production of hydrolysates, gelatine, fertilizers, and feed for livestock; furthermore, in the leather industry in the leather production and in the production of meat-and-bone meal. Adipose tissues are employed in the production of biofuels, industrial lubricants, oils, soaps, and as a functional additive for cosmetic products **(Lee, Lee and Song, 2015; Cruz-Fernández et al., 2017; Sarbon, Badii and Howell, 2013)**. Keratin hydrolysates obtained from feathers are used as growth promoters, feed additives, and functional additives in cosmetic products **(Ockerman and Hansen, 2000; Barbut, 2015; Dikeman and Devine, 2014; Wan Omar and Sarbon, 2016)**. Other wastes aim for the production of biofuels, composting, anaerobic digestion, or the isolation of valuable substances contained in animal by-products **(Borowski and Kubacki, 2015; Alibardi and Cossu, 2016; Xiong et al., 2016)**. The best solution would be to eliminate waste. Even though this is unfortunately very difficult to achieve, the optimal waste management must be pursued. To use slaughter by-

products efficiently, several criteria are vital to be accomplished. Primarily, a process recycling such a material to produce new products must be developed. Equally important is to provide a sufficient amount of slaughter by-products in the locality of new products manufacture together with the appropriate technological and economical background. A potential market where to offer these products is also essential. One of the ideal solutions appears to be the processing of slaughter waste, such as chicken stomachs, into further protein products containing significant amounts of collagen, vitamins, and minerals **(Rafieian, Keramat and Shahedi, 2015; Lee, Lee and Song, 2015; Khalid et al., 2011)**. It is important to explain that in the countries of Central Europe (the Czech Republic, Slovakia, Poland, and Hungary) poultry stomachs are considered to be edible offal. However, in Western Europe and America, these animal tissues are not included in a diet and are generally regarded as a slaughter waste. A suitable alternative to the utilization of chicken stomachs is in collagen products of gelatines and hydrolysates possible to apply in the food, pharmaceutical, cosmetic and medical industry. This would facilitate the management of an undesired and unused slaughter waste **(Alao et al., 2017; Toldra, 2006; Schreiber and Gareis, 2007; Rousselot gelatin, 2019)**.

Poultry slaughtering produces by-products having extraordinary physico-chemical properties **(Ockerman and Hansen, 2000; Ferraro, Anton and Santé-Lhoutellier, 2016)**. The chicken stomach is a part of the digestive system functioning as a smooth muscle bag divided into a muscular and glandular part. Only the muscular part is edible. The chicken stomach represents about 3% of the total weight of poultry. Since stomachs contain a significantly large amount of collagen, suitable methods of extracting gelatine from them have been investigating. Regrettably, chicken stomachs are composted or incinerated rather than used for the consumption in these regions so far **(Marvan, 2017; Huda et al., 2013; Kosseva, 2013).** The viscera, including chicken stomachs, provides extraordinary nutritional value and is highly appreciated in many parts of the world, such as in China, Japan, and India **(Bakar and Harvinder, 2002)**.

In practice, type A and type B gelatines are encountered. Type A gelatine is obtained by acid treatment of the raw material, while type B gelatine is extracted using a base. Currently, the extraction is performed using beef and pork skins and bones. This study examines the gelatine extraction after the prior enzyme treatment which seems to be the most convenient method of obtaining gelatine in terms of time and energy savings. Type B gelatine is treated for up to 6 months, type A gelatine for up to 40 hours, but enzyme extracted gelatine is treated for only up to 24 hours.

What is more, this form of gelatine is considerably well digested and absorbed in the gastrointestinal tract **(GMIA Standard Testing Methods for Edible Gelatin, 2019; Schreiber and Gareis, 2007; Mokrejš et al., 2019)**.

The aims of this study

As chicken stomachs are solid poultry by-products containing large amounts of collagen **(Marvan, 2017; Ockerman and Hansen, 2000)**, this study is to contribute to the investigation of suitable methods for the collagen extraction from such a slaughter waste. To our best knowledge, extraction and application of gelatine obtained from chicken stomachs by enzymatic treatment of the raw material have not been reported yet. Therefore, the aim of this paper is to assess the possibilities of extracting gelatine from chicken stomachs after the preceding biotechnological treatment of tissues with the proteolytic enzyme Protamex. It continues in the previous research "Preparation of protein products from collagen-rich poultry tissues" and "Utilization of protein by-products from poultry slaughterhouses for the preparation of collagen" **(Polaštíková et al., 2019a; Polaštíková et al., 2019b)**. This study focuses on monitoring the influence of selected technological conditions on the process efficiency and the final quality of extracted gelatine. The examined factors include the amount of the added Protamex proteolytic enzyme (Factor A; 0.1, 0.25, and 0.4%) and the extraction temperature (Factor B; 60, 62.5, and 65 ° C). Furthermore, it characterizes prepared gelatine by its gel strength and ash content.

Scientific hypothesis

Gelatine with a high gel strength of approximately 200 – 300 Bloom can be extracted from chicken stomachs.

MATERIAL AND METHODOLOGY

Material

Chilled chicken stomachs were provided by Raciola Uherský Brod, the Czech Republic. Stomachs were minced and homogenized to the particle size of 3 mm. The dry matter content was 19.10 ±0.05% and the composition in dry matter was as follows: protein content of 75.6 ±0.8%; fat content of 21.70 ±0.01% and mineral content of 3.900 ±0.005%.

Appliances, tools and chemicals

P-22/82 meat mincer Braher (Brather Internacional, Spain), LT2 shaker Kavalier (Kavalier, Czech Republic), Kern 440 – 47 electronic analytical scale, Kern 770 electronic analytical scale (Kern, Germany), pH meter Multical pH 526 (WTW, Weilhein, Germany), heating block LTHS 250 and 500 (Merci, Czech Republic), WTB Binder E28-TB1 driver (Binder, Germany), Memmert ULP 400 drying device (Memmert GmbH + Co. KG, Germany), SLR heating board with a magnetic stirrer (Schott Gerate GmbH, Germany), Stevens LFRA Texture Analyser for measuring gelatine gel strength (Leonard Farnell and Co Ltd., England), magnetic stirrer IKA Labortechnik PCT Basic with a heating and magnetic stirrer (IKA-Werke, Germany), differential scanning calorimeter DSC 1 (Mettler-Toledo, Germany), Mora hot air oven (Mora, Czech Republic), Nabertherm L9/11 muffle furnace (Nabertherm, Germany), desiccator (Kavalier, Czech Republic), EBA 20 centrifuge including a rotor (Hettich, Germany), vertical mixer ETA 0010 New Line (ETA, Czech Republic), KRUPS grinder and Samsung refrigerator (KRUPS, Czech Republic).

Erlenmeyer flasks of the volume of 2 L and 0.5 L; 2 L PET bottles with a screw cap; 25 mL, 200 mL, 250 mL and
1000 mL graduated cylinder; Petri dishes; pipettes;

weighing bottles; low-density filter papers; metal sieves; sprays with distilled water; scissors; gel strength flasks; non-stick drying pads; PA fabric; silicon crucible; 1 mm and 2 mm metal sieves; laboratory spoons and sticks; beakers; laboratory tongs; self-closing PE bags; funnels; metal sheet and adhesive tape.

Enzym Protamex (*Bacillus* protease complex developed for the hydrolysis of food proteins; declared activity of 1.5 $AU.g^{-1}$), distilled water, 0.03 M and 0.06 M NaOH, 0.2 M HCl, acetone, chloroform, ethanol, the enzyme Lipolase. The enzyme was provided by the Danish company Novozymes and the all chemicals used were provided by the Czech company Verkon.

Factor analysis

Factor analysis refers to a trial method describing the effect of individual factors on the total yield. It is a more time-consuming optimization method sensitive to measurement errors. It provides an extensive range of information; it monitors the impact of several factors on the sample. Factor analysis enables to evaluate not only one factor but also a complex of factors affecting the studied sample. Factor schemes of 2^2 or 2^3 are the most common. The analysis is a matrix creating a combination of input values. And the number of experiments depends on the number of variables **(Antony, 2014; Erge and Zorba, 2018)**. In this study, a factor scheme of 2^2 was applied for the experiments, for two levels and two examined quantities. The factors were as follows: the amount of Protamex enzyme added (Factor A; 0.1, 0.25 and 0.4%) and the extraction temperature (Factor B; 60, 62.5, and 65 °C). The enzymatic treatment of the raw material and the extraction time were constant for all laboratory experiments, 30 h, and 2 h, respectively.

Testing of functional properties gelatines

The extraction efficiency was calculated according to the following equation:

$$HY = \frac{m_1}{m_0} . 100$$

$$GY = \frac{m_2}{m_0} . 100$$

$$\eta = HY + GY$$

Where:

HY is the hydrolysate yield (%), m_0 is the weight of the defatted raw material (g), m_1 is the weight of the hydrolysate, GY is the gelatine yield (%), m_2 is the weight of gelatine (g) and η is the total yield (%).

Gelatine analysis providing ash content and gel strength was performed according to the Standard testing methods for edible gelatine **(GMIA Standard Testing Methods for Edible Gelatin, 2019)**. The melting temperature of gelatine gel was determined using a differential scanning calorimeter (DSC). After weighing 15 – 30 mg of the sample onto the DSC aluminum dish, it was sealed with a lid. Subsequently, the sample was placed into the measuring cell together with the reference sample. First, the DSC dish was cooled to 5 °C and maintained at this temperature for 5 min. Then, the dish was heated at a heating rate of 5 °C/1 min to the final temperature of 50 °C. Afterward, it was cooled to the initial temperature of 5 °C following the cooling rate of 5 °C/1 min. The melting temperature reflected an endothermic peak during the sample heating **(Höhne, Hemminger and Flammersheim, 2003)**.

Preparation of chicken stomach gelatines

Preparation of pure collagen

The purpose was to remove non-collagenous proteins and fat from the raw material to obtain isolated collagen which was then processed in gelatine extraction. First, the raw material was washed in water which removed albumins from the raw material. The treatment in 0.2 M NaCl at the ratio of 1:6 for 1.5 h followed to remove globulins. Then, the treatment with 0.03 M NaOH at the ratio of 1:6 for 20 h removed glutelins. And finally, the treatment with the enzyme Lipolase (the amount of 5% enzyme) with water 1:10 for 3 days defatted the material. Afterward, the defatted tissue was dried at 35 ±1 °C in the oven for 24 h. Thereafter, solvent defatting of the material was performed using acetone at the ratio of 1:9 for 20 h. This was followed by grinding pure collagen on a vertical mixer to the particle size of 1 mm.

Extraction of gelatine from pure collagen

The purified raw material was mixed with distilled water at the ratio of 1:10 and gently shaken at room temperature for 45 min. Then, the pH was adjusted to 6.5 – 7.0. Subsequently, the Protamex enzyme was added in the amount following *Factor A*, which is 0.1% or 0.25% or 0.4% of the enzyme (Table 1). The enzymatic treatment time of 30 h was constant for all experiments. In the next step, the raw material was filtered through a metal sieve, which was provided with 3 layers of PA fabric, and washed thoroughly with water to inactivate the enzyme partially. The material was then subjected to gelatine extraction. First, the washed material was placed into a beaker and mixed with distilled water at the ratio of 1:8. Subsequently, it was heated to the temperature of 60 °C, 62.5 °C, or 65 °C following *Factor B*. After reaching a defined temperature, the gelatine was extracted for 2 h. Finally, 200 mL of gelatine solution was poured onto a 330 cm^2 sheet provided with a non-stick film and dried in an air circulation drier at the temperature of 45 ±1 °C for 2 days.

Table 1 provides the list of experiments including the technological conditions, process characterization, and the list of prepared gelatines following the factor scheme of 2^2.

Statistical analysis

The results of all experiments were processed in MiniTab® 17.3.1 software (Fujitsu Ltd., Tokyo, Japan) for Windows. The statistical significance of the investigated process factors within the observed limits was evaluated on the significance level of p = 95%. Factors with a value lower than α = 0.05 influenced the evaluated variables with a 95% significance. The lower the p value, the greater the influence of process factors on the sample. Subsequently, the coefficient of determination characterizing the quality of the regression model was established and the data was graphically expressed.

Table 1 Characteristics of the experiments defining technological conditions, process characterization and attributes of prepared gelatines.

Exp. No.	Factor A Enzyme addition (%)	Factor B Extraction temperature (°C)	Yield of hydrolysate, η_H (%)	Yield of gelatine (main fraction), η_G (%)	Yield of gelatine (minor fraction), η_G (%)	Total extraction efficiency, $\sum\eta$ (%)	Gel strength, F ±SD (Bloom)	Ash content, AC ±SD (%)
1	0.10	60.0	7.76	24.39	32.70	64.85	192 ±10	1.87 ±0.04
2	0.10	65.0	6.65	23.84	13.31	43.80	429 ±8	1.60 ±0.30
3	0.40	60.0	8.87	86.47	1.11	96.45	8 ±0	1.10 ±0.90
4	0.40	65.0	6.65	88.69	0.55	95.89	2 ±0	1.00 ±0.30
5	0.25	62.5	7.76	63.19	9.67	80.62	96 ±4	1.40 ±0.30

RESULTS AND DISCUSSION

The evaluated variables included the degree of conversion, i.e. the percentage of conversion of the raw material into collagen products, the degree of purity of the final products in terms of ash content, and the quality of the extracted gelatine expressed in gel strength in Blooms.

The equation (1) of total extraction efficiency was:

$$\sum\eta = 177 + 139.5\,A - 2.16\,B \qquad (1)$$

The amount of added enzyme performed a statistically significant (p = 0.035) influence on the total extraction efficiency, whereas the extraction temperature showed no statistical significance (p = 0.309); R^2 = 93.58%.

Figure 1 depicts the effects of factors A and B on the total extraction efficiency. It reveals that the overall yield is the least (less than 50%) with the enzyme addition of 0.1% and the extraction temperature of 65 °C. Conversely, the highest total efficiency of more than 90% was recorded with the enzyme addition of 0.4% and the extraction temperature of 60 and 65 °C. At the temperature of 62.5 °C, the yield declined below 90% again. In general, the total efficiency increases with a rising amount of added enzyme and growing extraction temperature. Thus, the highest efficiency of 96.45% was monitored when 0.4% enzyme was added and the extraction temperature was 60 °C; the lowest efficiency of 43.80% was determined with 0.1% added enzyme and the extraction temperature of 65 °C.

The yield of the gelatine extraction from chicken stomachs varied between 23.84 and 88.69%. **Du et al. (2013)** treated chicken and turkey heads in acetic acid and

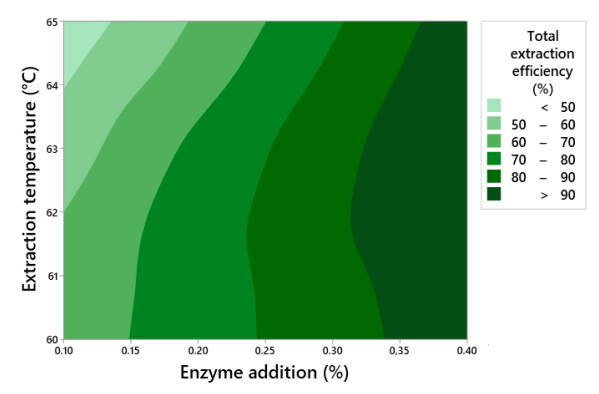

Figure 1 The impact of the amount of added enzyme and extraction temperature on the total extraction efficiency.

achieved gelatine yields ranging from 21.1 to 38.0%. Lower gelatine yield of 21.1% was obtained for chicken gelatine extracted at 60 °C and higher gelatine yield of approximately 38.0% was established for turkey gelatine extracted at 50 °C. In both studies, a lower gelatine yield was established if compared to the present experiment. **Almeida, Calarge and Santana (2013)** treated chicken feet at 120 °C for 20 min and extracted gelatine with a yield of about 36% which is in accordance with the yields determined in this study. **Cheng et al. (2009)** treated chicken feet in hydrochloric, acetic, and lactic acid and established the gelatine yield of 5.6 (HCl), 7.3% (acetic acid), and 8.3 (lactic acid) which is less than in this experiment. **Sarbon, Badii and Howell (2013)** extracted gelatine from chicken skin using both the acid and alkaline method with the total yield of only 16%. Therefore, it is evident that the acid and alkaline method may not be optimal to apply for skin processing. A higher yield of gelatine was achieved using the enzymatic treatment of the raw material **(Mrázek et al., 2019)**. Duck gelatine yield examined by **Huda et al. (2013)** was 28.4% which is a lower yield compared to the chicken gelatine yield of 31% achieved by **Liu, Lin and Chen (2001)**. **Abedinia et al. (2017)** treated duck feet using the acid, alkaline and enzymatic methods with the yields of 12.76, 11.39, and 17.94%, respectively. Even though their study confirmed the highest yield of gelatine by enzymatic treatment, it is still less than it was established in this experiment.

The equation (2) of gelatine gel strength was as follows:

$$F = -1044 - 1018\,A + 23.1\,B \qquad (2)$$

The amount of added enzyme and the extraction temperature did not show a statistically significant (p = 0.084; p = 0.346) influence on gel strength; R^2 = 85.69%.

Figure 2 depicts the impact of Factor A and B on gelatine gel strength. It is evident that to obtain high values of gelatine strength it is essential to apply higher extraction temperatures together with a lower amount of the enzyme. With 0.1% of the added Protamex enzyme and extraction temperature of 65 °C (Experiment 2), gelatine with the gel strength of more than 400 Bloom was extracted which is significantly high. Generally, the gel strength grows with a decreasing amount of enzyme and rising extraction temperature. In this study, it ranged from 2 ±0 to 429 ±8 Bloom. The lowest gel strength value was recorded with 0.4% of the added enzyme and at the extraction temperature of 65 °C. The highest values of gel strength

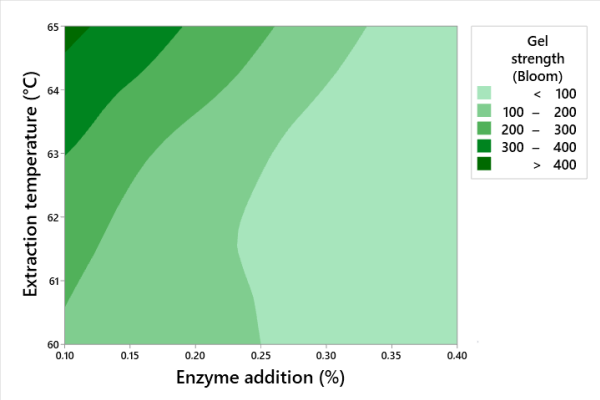

Figure 2 The impact of the amount of added enzyme and extraction temperature on the gel strength.

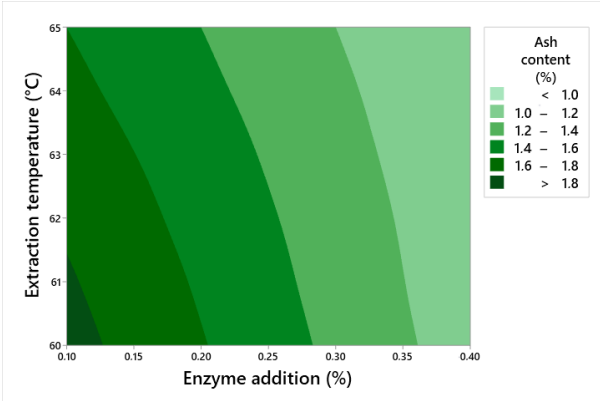

Figure 3 The impact of the amount of added enzyme and extraction temperature on the ash content.

were achieved in the extraction conditions of Experiment 2.

Du et al. (2013) extracted gelatine from turkey and chicken heads with a prior treatment in acetic acid and established the gel strength of 367 Bloom of turkey gelatine extracted at the temperature of 50 °C and the gel strength of 248 Bloom of chicken head gelatine extracted at the temperature of 60 °C which corresponds with this study. **Sarbon, Badii and Howell (2013)** stated bovine gelatine gel strength of 229 Bloom and chicken gelatine gel strength of 355 Bloom. High gel strength values ranging between 320 and 550 Bloom were established in the study by **Rafieian, Keramat and Kadivar (2013)**. **Rafieian, Keramat and Shahedi (2015)** examined chicken bone waste of mechanically deboned meat and determined the gel strength of 520 Bloom which exceeded the results of this experiment. **Sarbon, Badii and Howell (2013)** extracted chicken skin gelatine using both acidic and alkaline extraction methods and recorded the gel strength of 355 Bloom. Such a gel strength value confirms they have obtained the gelatine of considerably good quality. Compared to other alternative gelatine sources, such as fish, chicken gelatine achieves higher gel strength values; mackerel gelatine showed the gel strength of 280 Bloom and tilopia gelatine of about 220 Bloom **(Bakar and Harvinder, 2002)**. In the last decade, an interest in both poultry and fish gelatines has increased. Gel strength of fish gelatines may reach up to 420 Bloom. Such a significant gel strength was measured in gelatine extracted from tuna skin according to the study by **Zhou, Mulvaney and Regenstein (2006)**.

The equation (3) of the ash content in gelatine was as follows:

$$AC = -4.277 - 2.283\,A - 0.0370\,B \qquad (3)$$

For the ash content, the amount of added enzyme was statistically significant (p = 0.008). In contrast, the extraction temperature was statistically insignificant (p = 0.092); R2 = 98.58%.

Figure 3 shows the effects of Factors A and B on ash content. It is evident that to obtain a low amount of ash content in % it is vital to apply a lower/higher extraction temperature and a higher amount of the added enzyme. With 0.4% of the added enzyme Protamex and the extraction temperature of 60 and 65 °C, the ash content is approximately 1.1%. The ash content generally grows with a decreasing amount of the added enzyme and rising extraction temperature. The highest value corresponds with 0.1% of the added enzyme and the extraction temperature of 60 °C which reflects the extraction conditions in Experiment 1.

In the present study, the ash content ranged from 1.0 ±0.3 to 1.87 ±0.04%. **Du et al. (2013)** published a smaller ash content of only 0.03 to 0.06% in turkey and chicken gelatine extracted at 50 °C and 60 °C. **Almeida and Lannes (2013)** established the ash content in chicken feet gelatine of 1.9%. According to **The United States Pharmacopeial Convention (2018)** the maximal content of ash in gelatine must not exceed 2.0%; therefore, this factor has been accomplished in this study. **Bueno et al. (2011)** determined approximately 0.3% of ash in pork gelatine and **Sarbon, Badii and Howell (2013)** established 1.1% of ash in beef gelatine. In contrast to this study, **Rafieian, Keramat and Shahedi (2015)** recorded the ash content in chicken bone waste of 2.6%. **Sarbon, Badii and Howell (2013)** affirmed a lower ash content of 0.4% in chicken skin gelatine extracted using both acid and alkaline methods. **Huda et al. (2013)** extracted gelatine from duck feet using 5% lactic acid in the rate of 1:8 and established the ash content of 28.6% which is fourteen times higher than the required limit for gelatine application in the food industry.

Melting temperatures of gelatine gels

Figure 4 depicts DSC curve of gelatine gels melting temperatures. Experiment 4 (the gel strength of 2 ±0 Bloom) failed to identify the melting temperature of the gel since a hydrolyzate was formed. The gelatine of Experiment 1 (0.1% of the added enzyme and the extraction temperature
of 60 °C) performed a gelatine gel melting temperature of approximately 35 °C (the gel strength of 192 ±10 Bloom). Very similar melting temperature was achieved in Experiment 5 (0.25% of the added enzyme and the extraction temperature of 62.5 °C; the gel strength of 96 ±4 Bloom). The melting temperature of 36 °C was recorded in Experiment 3 with the gel strength of 8 ±0 Bloom (0.4% of the added enzyme and the extraction temperature of 60 °C). The highest melting temperature of gelatine gel with a strength of 429 ±8 Bloom (40.5 °C) was identified in Experiment 2 (0.1% of the added enzyme and

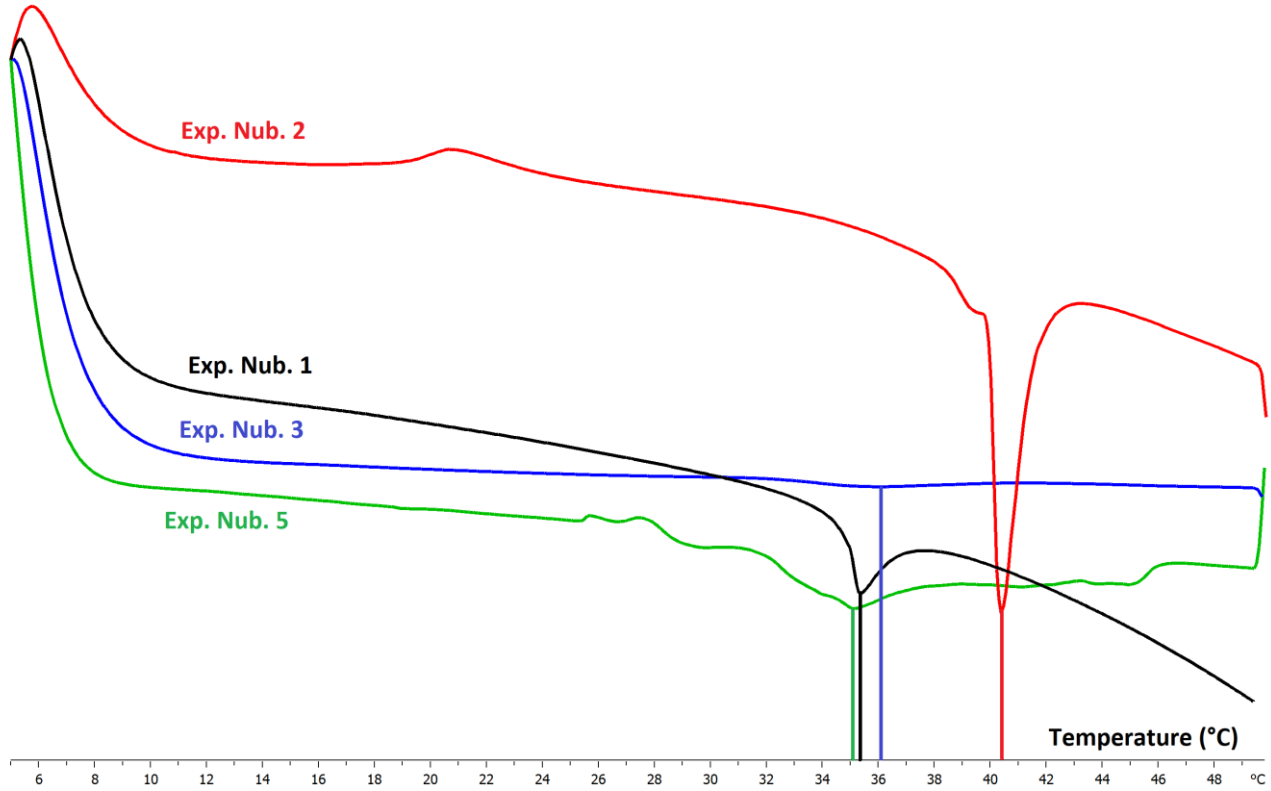

Figure 4 DSC curve of gelatine gel melting points.

the extraction temperature of 65 °C). Melting temperatures of commercial gelatine gels vary in the range from 30 to 40 °C. Their values are important not only from a technical point of view, but also considering the particular application of gelatines influencing various factors, such as the management of gelatine products, maintanance of the final shape of gelatine products and the stability of the products during the storage. Concerning gelatines extracted from chicken stomachs, their melting temperatures ranged from 35 to 40 °C which is comparable with commercial gelatines **(Schreiber and Gareis, 2007)**. **Du et al. (2013)** determined the melting temperature between 33.7 and 34.2 °C. That is slightly lower than the melting temperature of 35 – 40 °C established using DSC in this study reflecting the trend that melting temperature increases with a rising gelatine gel strength.

CONCLUSION

The study examines the possibility of extracting gelatine from chicken stomachs after the prior treatment by the proteolytic enzyme Protamex. The main objective was to propose technological conditions for processing stomachs into collagen products, either gelatines or hydrolysates, with a maximum yield. The influence of Factor A and B on the final efficiency and quality of extracted gelatine was monitored. Factor A represents the amount of added enzyme of 0.1, 0.25 and 0.4% and factor B represents the extraction temperature of 60, 62.5 and 65 °C. The extraction time of 2 h was constant. The final extraction efficiency ranged from 43.83% with 0.1% of added enzyme and the extraction temperature of 65 °C to 96.45% with 0.4% of added enzyme and the extraction temperature of 60 °C. The highest gel strength of about 430 Bloom was measured within the conditions of the enzyme addition of 0.1% and extraction temperature of 65 °C. On the other hand, the lowest gel strength of 2 Bloom was established with the enzyme addition of 0.4% and extraction temperature of 65 °C. The ash content in prepared gelatines was less than 2%; it ranged between 1.0 (0.4% of added enzyme and the extraction temperature of 65 °C) and 1.9% (0.1% of added enzyme and the extraction temperature of 60 °C). Edible gelatine with the gel strength of 96 Bloom (with the yield of 63%) is suitable for the applications in the production of confectionery, such as meringues, toffee, licorice and also deposited marshmallow. To produce jelly, gummy bears, aspic and dairy products it is preferable to employ gelatine with a higher gel strength (192 Bloom) despite its lower yield (approximately 24%). Both types of gelatine performed the ash content lower than 2.0% and the melting temperature of about 35 °C which means that such gelatines would be soluble in the mouth and simultaneously it would maintain the product shape during the storage, particularly during the summer months. Gelatine with a high gel strength of more than 220 Bloom is applicable in the production of desserts, extruded marshmallow, fish aspic and reduced fat spreads and in the pharmaceutical industry in the production of soft gelatine capsules.

This study has proved that it is possible to obtain high quality gelatine from chicken stomachs with the gel strength of up to 430 Bloom if appropriate technological conditions are set. The method applied in this study is quite prompt and efficient. Therefore, it has also confirmed that effective processing of valuable poultry slaughter by-products is accesible.

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SURVEY OF MYCOBIOTA ON SLOVAKIAN WINE GRAPES FROM SMALL CARPATHIANS WINE REGION

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ABSTRACT

A total of 13 samples of grapes (bunches) without apparent fungal contamination were analyzed. The samples were collected during the 2019 harvest from Vrbové village in the Small Carpathian region of Slovakia. For the isolation of fungi were used the direct plating technique on DRBC plates. The plates were incubated aerobically at 25 ±1 °C for one week in the dark. The data obtained from the cultivation of the grape berry samples revealed a high diversity of fungal species (a total of 1044 isolates were obtained). *Alternaria* and *Rhizopus* were the main components of the wine grape mycobiota of the Vrbovský subregion at harvest time (92%, each), followed by *Cladosporium* (85%), *Penicillium* (77%), *Botrytis* and *Epicoccum* (54%, each). The most abundant genera found by descending order were *Penicillium* (25%), *Alternaria* (24%), *Cladosporium* (20%), and *Rhizopus* (12%) and only in minor percentage by *Aspergillus* (3%) among others. The main fungal species isolated from genera *Penicillium* and *Aspergillus* were *Penicillium expansum* (57% RD) and *A.* section *Nigri* (97% RD). Of 17 analyzed *Penicillium* strains, 65% were able to produce at least one of the six mycotoxins analyzed in *in vitro* conditions by means of thin-layer chromatography method: citrinin, griseofulvin, patulin, cyclopiazonic acid, penitrem A, and roquefortin C.

Keywords: grape; filamentous fungi; *Penicillium*; mycotoxin; Slovakian vineyard

INTRODUCTION

Viticulture is an important activity in many countries **(Einloft et al., 2017)**. Vine growing and viticulture have a very long tradition in Slovakia and are parts of the country's cultural and historical heritage. Hundreds of years of viticulture and viniculture have created a specific type of landscape **(Bezák et al., 2010)**, with unique cultural and aesthetic values **(Salašová and Štefunová, 2009)**. In total there exist six viticultural regions in Slovakia with forty areas and wine-growing villages **(ÚKSÚP, 2019a)**. Slovakia features almost 660 producers growing around 13.500 ha of vines (of a potential 15.300 ha) **(ÚKSÚP, 2019b)** for a production of about 300.000 hL annually, which is primarily sold within the national market.

The microflora of the grapes is highly variable, mostly due to the influence of external factors as environmental parameters, geographical location, grape cultivars, and application of phytochemicals on the vineyards **(Pretorius, 2000; Pinto et al., 2014)**. A variety of fungal genera, mainly *Botrytis*, *Alternaria*, *Aspergillus*, *Penicillium,* and *Cladosporium*, can contribute to grape spoilage before harvest **(Bellí et al., 2006; Magnoli et al., 2003; Medina et al., 2005)**. Filamentous fungi impact negatively in the production, sensory quality, and safety characteristics of the wine in several ways. Their development in wine grapes brings significant yield losses for winemaking, alters the chemical composition of wine grapes, and produces secondary fungal metabolites and enzymes that together adversely affect wine flavor and color as well as yeast and lactic acid bacteria growth during vinification **(Fleet, 2003)**. Among them, it is of great concern the presence of toxicogenic fungi in wine grapes capable of producing mycotoxins that could persist during the winemaking process up to wine, being a high risk for consumer's health **(Paterson et al., 2018; Prendes et al., 2015)**.

The genus *Alternaria* is ubiquitously distributed and includes both saprophytic and opportunistic plant-pathogenic species, which may affect crops in the field or cause harvest and postharvest decay of plant products. Moreover, several *Alternaria* species are known to produce toxic secondary metabolites, *Alternaria* mycotoxins. The major *Alternaria* mycotoxins are the tetramic acid derivate, tenuazonic acid, and the dibenzopyrone derivates, alternariol (AOH), and alternariol monomethyl ether (AME) **(Prendes et al., 2015)**. Despite the toxic effects of the *Alternaria* toxins and their documented occurrence, they have not yet received the same attention as others mycotoxins and up to now, there is no regulation about them **(EFSA, 2011)**. As an opportunistic pathogen, it has the potential to cause a grape berry rot in the field under high disease pressure

situations. Strikingly, *Alternaria* has not been extensively studied in wine grapes as a hazardous genus.

P*enicillium* has gained attention as grapevine pathogens. *Penicillium expansum* can cause rot in grapes, but does not usually attack grapes before harvest. Aside from losses in fruit, this species is regarded as the major producer of patulin, although this species produces many other toxic metabolites such as citrinin, roquefortine C or chaetoglobosins among others **(Andersen, Smedsgaard and Frisvad, 2004)**.

Scientific hypothesis

Some of the fungal species occurring on grapes and grape products can produce mycotoxins, so species identification is critical to predicting the potential mycotoxin contamination of grapes and wine.

MATERIAL AND METHODOLOGY

Study area

Village Vrbové is located in the Vrbovský subregion in the Small Carpathian wine region. The Small Carpathian wine region is the most extensive of the six wine regions in Slovakia (vineyards are covering 4175 hectares) and is located in the southwestern part of Slovakia **(ÚKSÚP, 2019b)**. Vines have been grown on the south-facing slopes of the Small Carpathian mountains in locality Záhorie for more than three thousand years. This region has a medium climate and abundant moisture.

Last year, as a whole, was extremely warm. The year 2019 had the same average annual temperature in Hurbanovo 12.42 °C as in 2018. This value is a record high for Hurbanovo since the record began. During the whole year, 2019 was only one month of the territory temperature below normal. It was May **(Beránek and Faško, 2020)**.

Grape sampling

A total of 13 samples were taken: 3 from red varieties (Alibernet, Cabernet Sauvignon, and Blaufränkisch) and 10 from white varieties (Palava, Green Veltliner, Seteasca Regala, Chardonnay, Rheinriesling, Welschriesling, Sauvignon, Pinot Blanc, Irsai Oliver, and Müller Thurgau). The sampling was conducted at the 2019 vintage, at the end of September. Two diagonals crossing the vineyards were delimited, and five healthy and undamaged bunches from each diagonal were obtained. Each bunch was collected in a sterilized plastic bag and sent to the laboratory chilled on ice.

Mycological analysis

Fifty berries were selected randomly from each sample (totaling 650 berries) and placed in Dichloran Rose Bengal Chloramphenicol agar (DRBC) **(Samson et al., 2002)**. Plates were incubated at 25 ±1 °C for 7 days in darkness. Genera identification was conducted according to microscopic and macroscopic criteria using the key of **Pitt and Hocking (2009)**. *Aspergillus* strains were isolated and cultivated on MEA (Malt extract agar) **(Samson et al., 2010)**, CYA (Czapek yeast extract agar) **(Samson et al., 2010),** and CY20S (Czapek yeast extract with 20% sucrose) **(Pitt and Hocking, 2009)**. The *Aspergillus* colonies were identified to species level according to micro and macroscopic criteria, using the keys of **Klich (2002)** and **Pitt and Hocking (2009)**. *Penicillium* strains were isolated and cultivated on MEA, CYA, Creatine-Sucrose agar (CREA) **(Samson et al., 2010)** and Yeast Extract agar (YES) **(Samson et al., 2010)**. The *Penicillium* colonies were identified to species level according to **Pitt and Hocking (2009)** and **Samson and Frisvad (2004)**.

Mycotoxin production

Toxinogenity of selected isolates was screened in *in vitro* conditions by means of thin-layer chromatography (TLC) according to **Samson et al. (2002)**, modified by **Labuda and Tančinová (2006)**. Extracellular metabolites – citrinin griseofulvin and patulin were carried out on YES agar and intracellular cyclopiazonic acid, penitrem A, and roquefortin C on CYA agar. At 14 days of incubation, five agar plugs (4 mm diameter) were cut from the edge of a colony (extracellular metabolites) or cut from a colony (intracellular metabolites) from each Petri plate and placed in an Eppendorf tube. The plags were extracted in 500 µL of chloroform-methanol (2:1, v/v) (Reachem, Slovak Republic). The content of the tubes was stirred for 5 min by Vortex Genie ® 2 (MO BIO Laboratories, Inc. – Carlsbad, CA, USA). The extract of liquid phase 30 µL along with 10 µL of standards (Sigma, Germany) was transferred to the TLC plate (Alugram ® SIL G, Macherey – Nagel, Germany). The plate was put into TEF solvent (toluene:ethyl acetate:formic acid – 5:4:1, toluene – Mikrochem, Slovak Republic; ethyl acetate and formic acid – Slavus, Slovak Republic). After elution, the plate was air-dried. The identification of the metabolites was done by comparison with metabolite standards. Cyclopiazonic acid was visible directly in daylight after spraying with the Ehrlich reagent as a violet-tailed spot. Penitrem A was visible after spraying with 20% $AlCl_3$ in 60% ethanol and heating at 130 °C for 8 min as a dark blue spot. Roquefortin C was visible after spraying with $Ce(SO_4)_2$ x 4 H_2O as an orange spot. Patulin detection was achieved by spraying with 0.5% methylbenzothiazolone hydrochloride (MBTH) (Merck, Germany) in methanol and heating at 130 °C for 8 min and then detected as a yellow-orange spot under visible light. Citrinin was detected directly as an intense yellow-green streak under ultraviolet light (365 nm) as well as griseofulvin, which was visible as a blue spot.

Statistical analysis

The obtained results were evaluated and expressed according to relative density (RD) and isolation frequency (Fr). The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample **(Guatam, Sharma and Bhadauria, 2009)**. These values were calculated according to **González et al. (1999)** as follows:

RD (%) = (ni/Ni) x 100

where ni – number of isolates of a species or genus; Ni – total number of isolated fungi.

The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at

least once. These values were calculated according to **González et al. (1999)** as follows:

Fr (%) = (ns/N) x 100; where ns – number of samples with a species or genus; N – total number of samples.

RESULTS AND DISCUSSION

Fifteen fungal genera were identified from the grape samples: *Alternaria, Aspergillus, Aureobasidium, Botrytis, Cladosporium, Epicoccum, Fusarium, Mucor, Penicillium, Phoma, Rhizopus, Syncephalastrum, Trichoderma, Trichothecium,* and *Ulocladium*. About 2 % of the isolated fungi did not produce conidiophores or conidia on the tested conditions and were nominated as non-sporulated fungi *Mycelia sterilia*. The number of isolates within the several genera found on grapes from different varieties are shown in Table 1. The highest number of isolates (from 101 to 199) with 7, 8, or 9 genera were isolated from varieties Müller Thurgau (13), Irsai Oliver (12), Blaufränkisch (3), Palava (4), and Alibernet (1). The lower number of isolates (26, 28, respectively) were isolated from the white variety Rheinriesling (8) with the number of genera 6 and Pinot Blanc (11) with the number of genera 4. It is interesting to note the absence of isolates belonging to microscopic filamentous fungi in one sample Green Veltliner (5). This wine grape was colonized only by yeasts. All samples (except sample 5) were colonized by genera *Alternaria* and *Rhizopus*. Genus *Alternaria* was dominated in samles Palava (4), Cabernet Sauvignon (2) and Welschriesling (9), genus *Rhizopus* in sample Alibernet (1), genus *Penicillium* in samples Müller Thurgau (13), Irsai Oliver (12), Blaufränkisch (3) and Welschriesling (9) and genus *Cladosporium* in samples Irsai Oliver (12) and Sauvignon (10).

Thirty of the 32 *Aspergillus* isolates were identified as *A.* section *Nigri* and 1 isolate as *A. ochraceus.* Sixteen black aspergilli were isolated from the Blaufränkisch variety (3), 6 from Cabernet Sauvignon (2), 5 from Irsai Oliver (12), 2 from Palava (4), and 1 from Chardonnay (7). Among section *Nigri, A. carbonarius* is considered the predominant species responsible for the occurrence of OTA in wine grapes and derivatives **(Ponsone et al., 2010; Visconti et al., 2008)**. A low occurrence of this fungus was previously reported in Argentina **(Chiotta et al., 2009; Ponsone et al., 2010),** Brazilia **(Einloft et al., 2017)** and Lebanon **(El Khoury et al., 2006)**, and the absence of this fungus was observed in cold regions, like Germany, North Hungary, Czech Republic and Portugal **(Abrunhosa et al., 2001; Ostrý et al., 2007; Varga et al., 2005)**.

Three species of *Penicillium* were isolated from grapes. Species *Penicillium expansum* were dominated from the Müller Thurgau variety (13), Blaufränkisch (3), Seteasca Regala (6), Rheinriesling (8), and Palava (4). *Penicillium expansum* has a high incidence in certain wine regions such as bordering regions of North Portugal and Galiza (Spain) **(Serra et al., 2006)**. The incidence of *P. expansum* in some wine regions is high, but the attack of this fungus to vineyards, is rare, being *B. cinerea* the most common disease. **Morales et al. (2013)** observed that, *in vitro*, the presence of *P. expansum* spores enhanced *B. cinerea* growth, while the latter avoided patulin accumulation.

The data in Table 2 obtained from the cultivation of the berry samples revealed a high diversity of fungal species (a total of 1044 isolates were obtained). *Alternaria* and *Rhizopus* were the most frequently occurring genera (92%, each), followed by *Cladosporium* (85%), *Penicillium* (77%), *Botrytis,* and *Epicoccum* (54%, each). *Penicillium* spp. was predominant in terms of relative abundance (25%), followed by *Alternaria* (24%), *Cladosporium* (20%), *Rhizopus* (12%), and *Botrytis* (6%). Besides, a minor portion (<5%) of *Aspergillus* and other genera was found.

Alternaria genus was the main component of the wine grape mycobiota of the Vrbovský subregion (Small Carpathian wine-growing region) at harvest time, which is in agreement with previous studies carried out in several winemaking regions worldwide, e.g. from Uruguay **(Garmendia and Vero, 2016)**, Argentina **(Magnoli et al., 2003; Prendes et al., 2015)**, Spain **(Medina et al., 2005)**, Slovakia **(Felšöciová et al., 2015c; Felšöciová, Mašková and Kačániová, 2018; Felšöciová and Kačániová, 2019a; Felšöciová and Kačániová, 2019b)**.

It was followed by *Penicillium*, which recorded a frequency of 77% and a high relative density of 25%. From the previous study by **Felšöciová and Kačániová (2019a)**, *Penicillium* contributed a small proportion (21% Fr, <1% RD) from mycobiota associated with grapevine in Vrbové. The *Botrytis* genus, which is regarded as the main spoilage cause in wine grapes, was isolated in this study, but the absence of this genus has already been reported by **Magnoli et al. (2003)** in Argentina, and **Medina et al. (2005)** in Spain. Grey mold *Botrytis cinerea* is responsible for severe economic loss. Musts obtained from botrytized grapes are more liable to oxidation because of the polyphenol oxidizing activity of *B. cinerea* laccase and are not suitable for wine production **(Morales et al., 2013)**.

Aspergillus was one the less common genera (46% Fr, 3 % of all fungi). These results differ from those obtained by other authors, who reported a much higher frequency from this genus, ranging from 70% to 95% **(El Khoury et al., 2008; Magnoli et al., 2003; Medina et al., 2005)**.

Data in Table 3 show that, 32 *Aspergillus* species were identified from grape samples. The section *Nigri* was predominant within the *Aspergillus* genus, representing 94% of species isolated from this genus with 38% frequency. Certainly, the *Aspergillus* species are present worldwide, in all the grape products and under all environmental conditions **(Somma, Perrone and Logrieco, 2012)**. From the 12 vineyards in the Small Carpathian area (14 samples), 79% of the samples were coloniezed by the genus *Aspergillus* **(Felšöciová et al., 2015c)**. During the 3 years survey (2011, 2012, and 2013), 37 isolates belonging to 7 *Aspergillus* species (*A. clavatus, A. flavus, A.* section *Nigri, A. ostianus, A. parasiticus, A. versicolor* and *A. westerdijkiae*) were isolated. The main occurring *Aspergillus* species of the samples were *A.* section *Nigri* (64%), as in our research. On the other hand, the most species were not been isolated from any of the samples analyzed in the present study.

Table 1 Fungi identified in Slovak wine grapes from exogenous mycobiota in 2019 by direct plating method.

Fungal taxa	Grape varieties												
	1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**	**13.**
Alternaria	17	28	36	77	-	13	10	7	25	7	2	4	26
Aspergillus	-	**7**	16	2	-	-	1	1	-	-	-	5	-
A. ochraceus	-	1	-	-	-	-	-	-	-	-	-	-	-
A.* section *Nigri	-	6	16	2	-	-	1	-	-	-	-	5	-
***A.* sp.**	-	-	-	-	-	-	-	1	-	-	-	-	-
Aureobasidium	-	-	-	-	-	-	-	-	-	-	-	-	1
Botrytis	17	8	-	-	-	-	10	-	-	7	6	4	12
Cladosporium	14	7	14	2	-	5	5	-	6	22	9	67	55
Epicoccum	5	3	1	-	-	3	-	-	-	1	-	6	5
Fusarium	2	-	2	12	-	-	-	-	-	1	-	-	-
Mucor	-	4	-	3	-	1	4	2	-	-	-	3	-
Penicillium	19	5	48	5	-	12	-	10	23	1	-	51	90
P. crustosum	-	-	-	-	-	-	-	-	-	-	-	-	1
P. expansum	-	-	41	5	-	12	-	10	6	-	-	15	61
P. griseofulvum	-	-	-	-	-	-	-	-	-	-	-	10	-
***P.* sp.**	19	5	7	-	-	-	-	-	17	1	-	26	28
Phoma	1	-	-	-	-	-	1	-	1	-	-	-	-
Rhizopus	26	23	6	6	-	6	2	1	15	3	9	18	8
Syncephalastrum	-	-	-	-	-	-	-	-	-	1	-	-	-
Trichoderma	-	-	1	-	-	2	5	-	-	-	-	1	2
Trichothecium	-	-	-	-	-	-	-	1	-	-	-	-	-
Ulocladium	-	-	-	2	-	1	-	-	-	-	-	-	-
Mycelia sterilia	-	-	-	-	-	9	4	4	2	2	2	2	-
Total isolates	**101**	**85**	**124**	**109**	**-**	**52**	**42**	**26**	**72**	**45**	**28**	**161**	**199**

Note: 1. Alibernet, 2. Cabernet Sauvignon, 3. Blaufränkisch, 4. Palava, 5. Green Veltliner, 6. Seteasca Regala, 7. Chardonnay, 8. Rheinriesling, 9. Welschriesling, 10. Sauvignon, 11. Pinot Blanc, 12. Irsai Oliver, 13. Müller Thurgau.

Table 2 The occurrence, isolation frequency and relative density of filamentous microscopic fungi in surface mycobiota of grapes (n = 13) harvested in Small Carpathian region.

Fungal taxa	No.	Fr (%)	RD (%)
Alternaria	252	92	24
Aspergillus	32	46	3
Aureobasidium	1	8	<1
Botrytis	64	54	6
Cladosporium	206	85	20
Epicoccum	24	54	2
Fusarium	17	31	2
Mucor	17	46	2
Penicillium	264	77	25
Phoma	3	23	<1
Rhizopus	123	92	12
Syncephalastrum	1	8	<1
Trichoderma	11	38	1
Trichothecium	1	8	<1
Ulocladium	3	15	<1
Mycelia sterilia	25	54	2
Total isolates	1044		

Note: No – number of isolated micromycetes, Fr – isolation frequency, RD – relative density.

Table 3 The occurrence, isolation frequency and relative density of *Aspergillus* species in surface mycobiota of grapes (n = 13) harvested in Small Carpathian region.

***Aspergillus* species**	**No.**	**Fr (%)**	**RD (%)**
A. ochraceus	1	8	3
A.* section *Nigri	30	38	94
***A.* sp.**	1	8	3
Total isolates	**32**		

Note: No. – number of isolates, Fr – isolation frequency, RD – relative density.

Table 4 The occurrence, isolation frequency and relative density of *Penicillium* species in surface mycobiota of grapes (n = 13) harvested in Small Carpathian region.

***Penicillium* species**	**No.**	**Fr (%)**	**RD (%)**
P. crustosum	1	8	<1
P. expansum	150	54	57
P. griseofulvum	10	8	4
***P.* sp.**	103	54	39
Total isolates	**264**		

Note: No. – number of isolates, Fr – isolation frequency, RD – relative density.

Table 5 Toxinogenity of selected *Penicillium* strains, isolated from exogenous mycobiota of wine grapes.

Species	**C**	**G**	**P**	**CA**	**PA**	**RC**
P. crustosum					1/1	0*/1**
P. expansum	13/14		11/14			9/14
P. griseofulvum		2/2	2/2	1/2		1/2

Note: * – number of isolates with ability to produce mycotoxin, ** - number of tested isolates, C – citrinin, G – griseofulvin, P – patulin, CA – cyclopiazonic acid, PA – penitrem A, RC – roquefortin C.

Data in Table 4 show 3 different *Penicillium* species from the 264 fungal strains. *Penicillium expansum* was predominant within the *Penicillium* species, representing 57% of the isolates and 54% frequency, which agrees with previous publications by **Felšöciová and Kačániová (2019a)**, **Felšöciová and Kačániová (2019b)**. The predominant species of *Penicillium* isolated from grapes differs between vineyards. For example, *Penicillium expansum* is the species most frequently isolated in South Slovak wine region (28% RD) **(Felšöciová et al., 2017)**, *P. aurantiogriseum* in East Slovak wine region (34% RD) **(Felšöciová et al., 2015a)**, *P. chrysogenum* in Small Carpathian wine region (64% RD) **(Felšöciová et al., 2015c)**, in Nitra wine region (28% RD) **(Felšöciová et al., 2013)**, in Central Slovak wine region (53% RD) **(Felšöciová et al., 2014)** and Tokaj (39% RD) **(Felšöciová et al., 2015b)**. *Penicillium expansum* was found frequently in botrytized grapes **(Morales-Valle et al., 2011)**. This species was the second frequent (after *P. chrysogenum*) in Tokaj (33% RD).

The toxicogenic profile of the 17 *Penicillium* isolates representing *P. crustosum, P. expansum* and *P. griseofulvum* from the Slovak grapes is shown in Table 5.

The 65% of the 17 analyzed *Penicillium* strains were able to produce at least one of the six mycotoxins tested (citrinin, griseofulvin, patulin, cyclopiazonic acid, penitrem A, and roquefortin C). Citrinin was the toxin produced by the majority of the strains *P. expansum* (93%). It was followed by patulin produced by 79% of the strains *P. expansum*, and roquefortin C produced by 64% of the strains. *Penicillium crustosum* produced only penitrem A, did not produce roquefortin C. Two strains of *Penicillium griseofulvum* produced griseofulvin and patulin, the production of cyclopiazonic acid and roquefortin C was confirmed by one isolate.

Almost 100% of *Penicillium expansum* strains are patulin producers **(Andersen, Smedsgaard and Frisvad, 2004; Morales et al., 2008)**, which does not fully correspond to our results. *Penicillium expansum* is commonly associated with apple rot, production of geosmin − a well-known compound with a strong earthy smell, and patulin contamination in apple derivatives **(Morales-Valle et al., 2011)**. However, patulin has been reported in grapes **(Moake, Padilla-Zakour and Worobo, 2005)**, processed grape juice **(Scott, Fuleki and Harwig, 1977),** and fermenting wine **(Majerus, Hain and Kölb, 2008; Bragulat, Abarca and Cabañes, 2008)**, although the occurrence in wine is low because it is well-known to be degraded partially by the fermentation process **(Moss and Long, 2002)**. Patulin mainly induces gastrointestinal disorders including ulceration, distension, and bleeding. The compound provokes congestion and oedema of pulmonary, hepatic, and gastrointestinal blood vessels and tissues. Subcutaneous injection of patulin produced local sarcomas in rats and is classified in group 3 as not classifiable as to its carcinogenicity to human by IARC **(Varga et al., 2015)**.

CONCLUSION

Our results indicate a high diversity of fungal species with a high incidence of *Alternaria* genus. Out of 17 potentially toxigenic *Penicillium* strains isolated from exogenous mycobiota, namely *P. crustosum, P. expansum* and *P. griseofulvum*, 65% produced at least one mycotoxin by thin-layer chromatography method. The occurrence of the potentially toxigenic fungus *Aspergillus* was overall very low what indicates the high quality of the wine grapes produced in Slovakia.

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SENSORY ACTIVE SUBSTANCES CAUSING OFF-ODOR IN LIQUID WHEY DURING STORAGE

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ABSTRACT

Liquid whey is a nutritious product with high water activity and neutral pH. Therefore, it is very susceptible to microbiological spoilage that results in undesirable off-odors. Additionally, minimally processed foods are the recent trend so setting an appropriate shelf life is essential. The commonly used microbiological methods are lengthy and time-demanding, so a quick and early identification of microbial degradation would be a significant benefit. Here we tested a solid-phase microextraction, gas chromatography with mass spectrometry coupled with olfactometry analysis (SPME-GC-MS/O) on samples of sweet unpasteurized liquid whey stored at 6 °C, 12 °C and 25 °C for a week. We compared the common methods – plate methods, measurement of pH, and dry matter determination with our proposed SPME-GC-MS/O. We have identified seven sensory active compounds while octanoic acid and a compound not reliably identified by the MS detector (with main m/z observed 133 (100), 151 (65), and 135 (26)) being the most prominent. Microbiological methods proved irreplaceable for proper setting of storage conditions (with the growth of coliforms being significant (p <0.001) at 25 °C). However, SPME-GC-MS/O was able to identify volatile substances responsible for off-odors and can be used as a powerful tool to detect the cause of undesirable chemical and microbial changes in whey beverages.

Keywords: whey; SPME-GC-MS/O; off-odor; analysis

INTRODUCTION

Sensory characteristics, such as appearance, taste, and aroma, are the basic parameters for evaluating the quality of many products. While traditional sensory analysis continues to be a valuable method of food and beverage analysis, it is not without its limitations in the evaluation of certain defects. Recently, we found this to be the case when presented with the problem of identification of the off-odor in real samples of liquid whey.

Whey is the leftover liquid when coagulating milk to produce cheese or the released liquid after the fermentation of other dairy products, most often Greek-style yogurts or skyrs. After the coagulation of milk with enzymatic rennet in the production of cheeses, sweet whey is produced, while the use of lactic acid in the production of curd results in acid whey. These two types differ slightly in their composition **(Karagul-Yuceer, Drake and Cadwallader, 2003)**.

While both types mostly consist of water, lactose, proteins, minerals, and fat, acid whey contains more minerals (especially calcium) and less proteins and lactose than sweet whey **(Kilara, 2015)**. Lactose makes up more than 75% of the total solids and is also the main reason why whey is considered as one of the most polluting food streams. On the other hand, the contained proteins and peptides (mainly α-lactalbumin, β-lactoglobulin, serum albumin, and immunoglobulins) are of exceptional biological and functional value and thus offer a wide range of whey utilization **(Anand, Khanal and Chenchaiah, 2013; Smithers, 2008)**.

Dried or concentrated whey and products where some ingredients, especially proteins, are isolated or concentrated (whey protein hydrolysate (WPH), whey protein concentrate (WPC, 34 – 89% protein) and whey protein isolate (WPI, >90% protein)) are widely used as a food ingredient for human consumption **(Evans et al., 2009)**. Liquid whey drinks are gaining popularity, either native, demineralized, or further processed (fermented, carbonated) and flavored in various ways **(Francis, 1999)**.

Liquid whey beverages are very susceptible to microbial spoilage and associated undesirable qualitative deviations because of their rich nutritional content and high water activity. Their shelf life is ensured either by heat treatment (pasteurization or sterilization) or by fermentation and subsequent cold storage **(Lo et al., 2016)**.

Microorganisms generally spoiling whey are the ones that typically spoil milk. Raw and pasteurized milk, exposed to secondary contamination, is most often contaminated by gram-negative bacteria of the genus *Pseudomonas*, while pasteurized milk is most often spoiled by thermoduric spore-forming microorganisms of the *Bacillus* and *Paenibacillus* species. Since whey is easily subjected to lactose fermentation to produce ethanol, acetic, lactic, and propionic acid (which is formed from

lactic acid by the bacteria of the genus *Propionibacterium*), the naturally present lactic acid bacteria – LAB (e.g, *Streptococcus, Lactobacillus* and *Lactococcus*) form a variety of aromatic active compounds such as 2,3-butanedione (diacetyl), acetoin, acetaldehyde or acetic acid from pyruvate, an intermediate in lactose fermentation **(Lo et al., 2016)**. When preparing alcoholic whey beverages, mainly yeasts of the genus *Kluyveromyces (K. fragilis* and *K. marxianus*) are used. These beverages are characterized by the presence of volatile compounds, including higher alcohols (mainly isoamyl alcohol, isobutanol, and 1-propanol), ethyl esters (mainly ethyl acetate), as well as acids and acetals **(Dragone et al., 2009)**. All of these substances contribute to the natural aroma of whey, but at higher concentrations to an undesirable odor. However, the main contributors to the off-odor of both liquid and dry whey are lipid oxidation products namely aldehydes, ketones, alcohols, and alkanes **(Carunchia Whetstine et al., 2003)**.

Sensory properties have traditionally been described and evaluated via sensory analysis, which can be loosely divided into two groups: discriminant methods and descriptive methods. The purpose of discrimination testing is to indicate whether a tested sample is perceived as being significantly different from a standard one (e.g. Triangle or Duo-Trio test). Descriptive methods, such as the flavor profile method or quantitative descriptive analyses, are more similar to chemical analysis in that they aim to determine the presence or intensity of a particular characteristic **(Kilcast, 2010)**. The problem is that while descriptive methods can characterize a particular off-odor, they are not able to link it to the specific compound, or compounds, responsible for certain occasional defects. That is where instrumental methods come in.

However, instrumental methods are best used in combination with sensory analysis. For example, GC-MS is able to identify the most abundant volatile compounds in a sample but cannot provide clear information on whether the substances are sensorially active. And the most sensitive physical detectors (MSD, ECD, FID) only have detection limits ranging from 1 to 10 pg, whereas human noses can readily detect to 0.05 pg **(Muñoz et al., 2010)**. Gas chromatography with an olfactometric detector (GC-O) combines the high resolution of capillary gas chromatography with the high selectivity and sensitivity of the human nose to detect and identify the compound, or compounds, responsible for an off-odor. The assessors sniff the eluate from the gas chromatograph using a special olfactory port to detect the presence of sensory-active compounds. Recently, solid-phase microextraction with gas chromatography/mass spectrometry coupled with olfactometry (SPME-GC-MS/O) has been used to identify substances in a variety of matrices, including coffee, cheeses, milk powders, orange juice, cashew apple (*Anacardium occidentale*) juice, yogurt, and even chocolate. **(Zellner et al., 2008; Gocmen et al., 2005; Semmelroch and Grosch, 1995; Zepka et al., 2014)**

Scientific hypothesis

The determination of sensory active substances allows for quick and early identification of microbial degradation and lipid oxidation.

MATERIAL AND METHODOLOGY

Samples

Samples of unflavoured, unpasteurized sweet liquid whey, with a fat content of up to 1%, sold in 1 liter PET bottles were purchased for the analysis. Recommended storage at a temperature from 4 °C to 8 °C and up to 4 days. Individual whey samples in the original packaging were analyzed (for pH, dry matter, and microbiology) 1 day after production (at time T0) and after 1 week stored in thermostats at 6 °C, 12 °C and 25 °C. This shelf-life study was conducted in two batches, and each sample was stored in each temperature in duplicates. Therefore, we obtained 4 sets of data for each storage temperature.

Methods

Microbiological

Samples were analyzed using plate methods **ISO 7218 (2007)** for coliforms according to **ISO 4832 (2006)** using VRB agar (Merck) and yeasts and molds according to **ISO 21527-1 (2008)** using YGC agar (Merck).

pH

The pH was measured using an Inolab pH meter (Thermo Scientific).

Dry matter

Samples were dried to a constant weight at 105 °C.

Volatile compounds

Volatile compounds were measured by SPME-GC-MS for samples T0, 6 °C and 25 °C under the same conditions as sensory active compounds.

Sensory active compounds

For the evaluation of sensory active compounds, the evaluators were first tested and trained by sniffing sticks (Olfasense GmbH), and the samples T0, 6 °C, and 25 °C were subsequently analyzed by SPME-GC-MS/O.

Testing by assessors

Ten assessors underwent two sets of tests. In the first one, the assessors were asked to match a sniffing stick to an odor written on the list. The second one was to describe the odor of each sniffing stick without using any prompts.
The batch of sniffing sticks included the following standards:

1. (E,E)-nona-2,4-dienal (fatty, rancid odour)
2. non-2-enal (paper, carton)
3. dimethyl disulphide (garlic, sulphur)
4. acetoin (yogurt)
5. methional (boiled potatoes)
6. δ-decalactone (floral, fruit)

Six assessors out of ten who showed low detection limits, low recognition thresholds and were in particular accurate in verbal identification of the unknown aroma of the standard compounds of sniffing sticks were subsequently involved in the olfactometric detection.

SPME-GC-MS/O

1 gram of sample was placed into a 10 mL vial. Determination of the volatile profile and sensory active substances was performed using Agilent GC 7890B, MS

5977A with DB-5 capillary column (30 m x 250 µm x 0.25 µm) and SPME fiber 50/30 µm DVB/CAR/PDMS (Supelco). The injector was operated in split mode 1:1, with He 5.5 flowing at 1.4 mL.min^{-1}. The temperature conditions were as follows: incubation for 60 s at 50 °C, with 1500 s sorption injector temperature was set at 260 °C with 360 s long desorption at 260 °C. GC system was set to 60 °C for 2 min followed by a temperature rise of 10 °C/min to a final temperature of 290 °C. NIST integrated library and its retention indices were used for the identification **(NIST MS Search 2.0)**.

The eluate was split 1:1 between the MS detector (MSD 230 °C, quadrupole 150 °C) and the olfactometer (JAS, 180 °C, capillary diameter 150 µm with the airflow rate 40 mL.min^{-1} of the humidifier) at the outlet of the GC column. Nasal impact frequency (NIF) technique with the posterior evaluation of odor intensity (1 – lowest intensity to 3 – highest intensity) was used. NIF value equals the number of assessors detecting a compound dived by the total number of assessors **(Plutowska and Wardencki, 2008)**.

Statistical analysis

The R Program (R Core team, 2017, version 3.5.2.) for Statistical Computing was used for statistical evaluation namely ANOVA and t-test. Results are presented as mean ±standard deviation

RESULTS AND DISCUSSION

The storage experiment was designed to copy the recommended storage conditions while promoting and accelerating the development of sensory active substances indicative of undesirable changes. For the samples stored at 6 °C for 1 week, there were no significant changes in coliforms, yeast and mold count, dry matter, and pH, as expected, because the declared storage conditions were 4 – 8 °C with a shelf life of 4 days (Figure 1 shows the average values of the 4 measurements). The whey dry matter content did not change significantly (p >0.05) with the storage temperature. At the same time in samples stored at 12 °C and 25 °C, the pH dropped significantly (p <0.001) by 35%. This pH drop was caused by an increased content of organic acids (probably produced by lactic acid or acetic acid bacteria and yeasts) **(Campbell et al., 2011; Sattin et al., 2016)**, which was confirmed by a subsequent chromatographic analysis of the volatile compounds. The number of coliforms and yeasts in the sample stored at 25 °C increased significantly (p <0.05) (coliforms one hundred times, yeasts almost five thousand times). No molds were detected in any samples.

The profile of volatile substances of fresh whey was very poor. Only hexane and octanoic acid had a peak area higher than 10^4. Other peaks were either not identified or were contaminants from the chromatographic system (siloxanes, higher hydrocarbons, etc.). The limited sensitivity of the MS detector is unfortunately due to a compromise of possible simultaneous application of olfactometry (see batch separation conditions and split between detectors).

After the storage experiment at 25 °C, the profile of volatiles changed drastically both in the number of peaks as well as in their area. A number of ketones (acetone, 2,3-butanedione, 2-butanone, acetoin, 2,3-pentandione, 2-heptanone, 2-nonanone), sulphur compounds (dimethyl sulphide dimethyl disulphide, dimethyl trisulphide, 2,4-dithiapentane), carbonyl compounds (heptane, hexane), an aldehyde (nonanal), alcohols (1-butanol 1-hexanol, 1-octanol) and organic acids (acetic acid, hexanoic acid, and octanoic acid) were found. We have not identified as many aldehydes as **Croissant et al. (2009)** but a wider range of compounds more similar to **Leksrisompong, Miracle, and Drake (2010)** and **Liaw et**

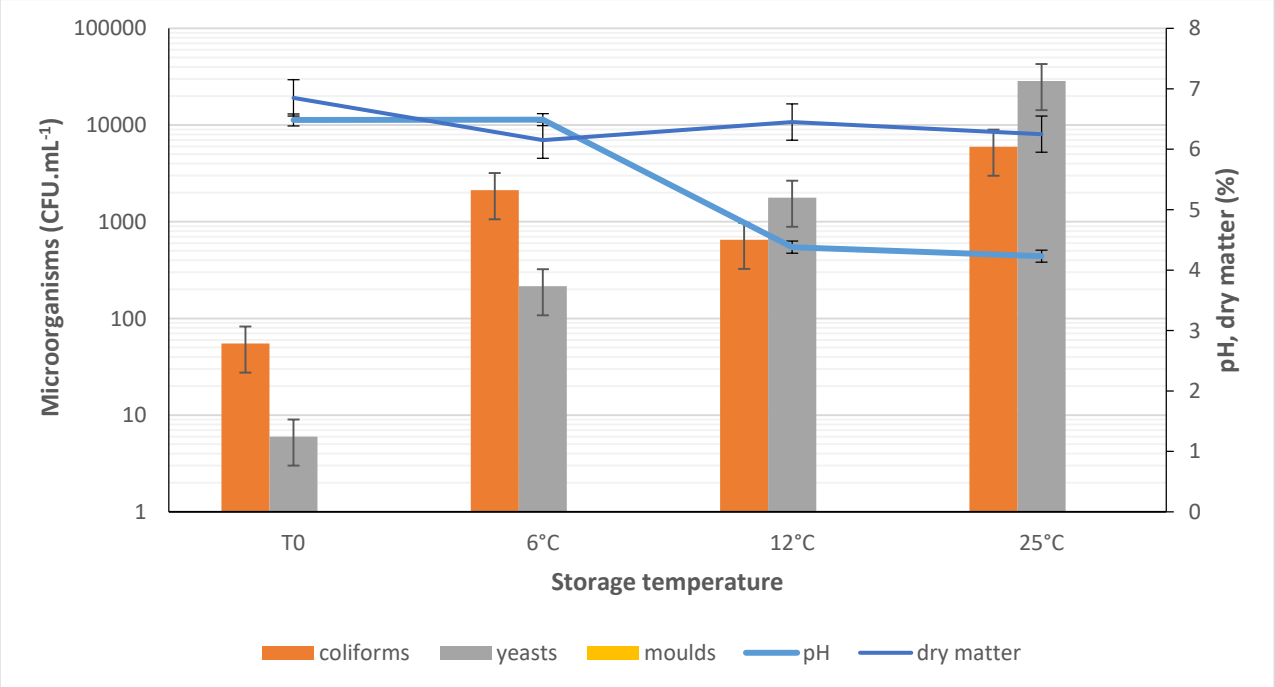

Figure 1 Microbiological, pH and dry matter analysis results obtained from 4 replicates of whey samples analysed at the time of purchase (T0) and after storage in 6, 12 and 25°C for 7 days.

Table 1 Sensory active substances detected by at least 2 assessors out of 6.

Rt	RI (NIST)	Compound	Odour (labelled)	Odour (perceived)	NIF*		
					T0	6 °C	25 °C
1.88	534	Dimethyl sulphide	Cabbage, onion, sulphur	Pungent	-	-	0.50
2.17	593	2,3-butanedione	Butter, caramel, cream, sweet	Butter, sweet, milk	-	-	0.67
5.16	801	Hexanal	Green, fatty, leafy	Fresh, grass, butter	-	-	0.67
7.43		NI		Cabbage, fatty, cheesy	1.00	1.00	1.00
8.52	982	Dimethyl trisulphide	Sulphur, onion, cooked	Cabbage, sulphur, cheese	-	-	0.83
8.71	1000	Hexanoic acid	Cheese, fatty, acid, sweet	Mushroom, fruity	-	-	0.67
11.89	1191	Octanoic acid	Cheese, fatty, sweet, rancid	Milky, musty	0.67	0.67	1.00

Note: *NIF value equals the number of assessors detecting a compound divided by the total number of assessors.

al. (2011) findings, including the not commonly found acetoin (a product of LAB metabolism) **(Nadal et al., 2009)**.

Compared to other dairy products, whey is not very rich in sensory active substances **(Fox et al., 2016; Qian and Reineccius, 2003)**. This finding is in an agreement with our measurements of fresh whey at T0 where only 2 sensory active compounds were detected – not identified (NI) compound and octanoic acid (Table 1). Octanoic acid was found to have a very low odor threshold in air (0.86 $\mu g.kg^{-1}$) **(Cometto-Muñiz and Abraham, 2010)** as opposed to, for example, hexanal (having 0.14 $\mu g.L^{-1}$ therefore 119 $\mu g.kg^{-1}$ in the air at 25 °C **(Ömür-Özbek and Dietrich, 2008)**. This explains why it was detected at T0, along with the fact that hexanal is an oxidation product from linoleic acid, therefore its concentration increases during storage. The other compound that could not be reliably identified by the spectra NIST library (the probability match was less than 50%) had 133 (100), 151 (65), and 13 5 (26) m/z ions as the largest. Detection of such a compound confirms the higher sensitivity of a human nose compared to a mass detector **(Muñoz et al., 2010)**. In total, seven compounds, mostly with an unpleasant odor, were detected by at least two assessors in the sample stored at 25 °C their odor was compared to literature ("The Good Scents Company," n.d.).

Dimethyl sulphide is usually associated with a cabbage-like odor produced by cooking certain vegetables and cereals, formed along with dimethyl trisulphide by bacterial degradation of sulphur amino acids **(Franco-Luesma and Ferreira, 2016; Luo et al., 2018; Nishibori et al., 2014)**. 2,3-butanedione, or diacetyl, is a natural by-product of the fermentation of lactic acid by the oxidative decarboxylation of α-acetolactate **(Hugenholtz et al., 2000)**. It may also be formed as an intermediate in high-temperature treatment with non-enzymatic Maillard browning and may later be involved in Strecker degradation with other free amino acids **(Smit, Smit and Engels, 2005)**. It is responsible together with acetoin for the characteristic taste of butter **(Karagul-Yuceer, Drake and Cadwallader, 2003)**. Hexanal is a lipid oxidation product and has been proposed as a potential quality marker. Free fatty acids (hexanoic and octanoic) are formed by microbial hydrolysis of fats **(Panseri et al., 2011)**.

Based on the assessor's results, dimethyl trisulphide produced the highest intensity odor, even though one assessor did not detect it. There are a number of factors that could have caused it, for example, selective anosmia to sulphur compounds (as dimethyl sulphide was not detected either) or the assessor's fatigue **(Brattoli et al., 2013)**. Dimethyl trisulfide had a peak approximately 20 times smaller than octanoic acid but since the peak area does not correlate with the odor intensity **(Högnadóttir and Rouseff, 2003)**, olfactometry was able to mark it as one of the main compounds responsible for off-odor.

CONCLUSION

When storing whey samples at elevated temperatures (12 °C and 25 °C), there was a significant increase (p <0.001) in the number of coliform bacteria and yeasts which led to an increased amount of organic acids and alcohols, causing undesirable off-odors. However, the scientific hypothesis (Determination of sensory active substances allows for quick and early identification of microbial degradation and lipid oxidation) is only partially confirmed. Since the slight (by 1 – 2 order) increase in the number of pathogenic and spoilage microorganisms at low

storage temperature was not yet reflected in the sufficient production of secondary volatile metabolites (and therefore on the sensory properties of the product) the SPME-GC-MS/O method was not able to detect it.

Thus we conclude that the traditional microbial testing is irreplaceable for a proper setting of storage conditions and shelf life Olfactometers can then play a significant role in detecting the causes of major product odor changes(as we have shown on the sample stored at 25 °C) and therefore spotting specific signs of microbial and chemical degradation.

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MEAT PERFORMANCE OF JAPANESE QUAILS AFTER THE APPLICATION OF BEE BREAD POWDER

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ABSTRACT
The aim of the study was the evaluation of meat performance of Japanese quails after the addition of bee bread powder into their diet. A total of 80 one day-old Japanese quails were randomly divided into 4 groups (n = 20): the control group (C) without additional supplementation, the experimental group E1 supplemented with 2 mg bee bread powder per 1 kg of feed mixture; the experimental group E2 supplemented with 4 mg bee bread powder per 1 kg of feed mixture and the experimental group E3 supplemented with 6 mg bee bread powder per 1 kg of feed mixture. The groups were kept under the same conditions and the quails were slaughtered at 56 days of age. Based on the results, we can conclude that the application of bee bread powder generally has not confirmed a positive effect on the meat performance of Japanese quails, regarding to the quantities of bee bread powder in the experimental groups.

Keywords: Japanese quail; meat performance; bee bread

INTRODUCTION
The poultry industry is considered to be one of the most advanced in the field of food industry and the increase in the production of poultry products has been remarkable. Today, products of different species of poultry, including quail products, are being marketed **(Genchev et al., 2008)**. Japanese quail (*Coturnix coturnix japonica*) is one of the most important species of poultry, which meat and eggs are mostly consumed in Asia, Europe, and America **(Minvielle, 2004; Kayang et al., 2004; Maiorano et al., 2012)**. The Japanese quail is characterized by its rapid growth, enabling quail to be marketed for consumption at 5 weeks of age, early sexual maturity resulting in a short generation interval, high rate of egg-laying and much much lower feed and space requirements than domestic **(Hrnčár et al., 2014)**. The valuable dietetic properties of quail meat are at the background of the increasing interest of consumers in this product. Quails can be used for meat production within a short period (4 – 5 weeks) and mature at an early age of 6 weeks so that female birds are usually in full production at approximately 8 weeks **(Jatoi et al., 2013)**. Japanese quails respond very quickly to the selection for higher body weight. **Anthony, Nestor and Marks (1996)** observed that some selected lines of Japanese quail produced heavier carcasses and more meat. The analysis of efficiency of quail meat production showed that it was the highest if the slaughtering was performed at 35 days of age **(Kajtazov and Genchev, 2004)**. The percentage content of edible meat in Japanese quail is very high: breasts ranging 37.3 – 38.7% of the body, legs 22.7 – 24.6% and the carcass, neck and wings in total 35.9 – 37.8% **(Panda and Singh, 1990; Alkan et al., 2010)**. Boned meat of the valuable parts of the body (breasts and legs) amounts to 36% for the breasts and 15% for the legs **(Vaclovsky and Vejcik, 1999)**.

The major advantage of quail rearing is that it requires minimum space, less capital investments and shorter generation interval. Furthermore, they are characterized by their early sexual maturity, better disease resistance, better feed efficiency and faster growth rate **(Vali, 2008)**. The average weight of a Japanese quail is 250 g and lays 250 eggs per annum. It is the smallest avian species reared for egg and meat purposes. Quail meat possesses low number of calories with high protein content. The average dressed carcass yield is 65 – 70% **(Krishnan, 2019)**.

A huge amount of antibiotics has been used to control diseases and to improve performances in livestock. Antibiotics are microbial metabolites that can inhibit the growth of other microorganisms even in low concentrations **(Nir and Ve-Senkoylu, 2000)**. But by long-term use, may cause some side effects of antibiotics, to occure residues in meat and the development of drug-resistance bacteria and reduction in the ability to cure these bacterial diseases in humans **(Donoghue Dan, 2003)**. To meet consumers' demands, in 2006, the European Union introduced a total ban regarding to usage of feed antibiotics. However, a ban on the use of antibiotics, as growth promoters, has led to a need for finding additives, yet safe for improving production performances without

negative effects on animal health and welfare, the quality of food of an animal origin, human health and the environment **(El-Medany et al., 2017)**. Bee products seem to be an effective natural alternative to antibiotic growth promoters **(Babaei et al., 2016; Haščík et al., 2016a, Haščík et al., 2017)**. Bee bread (ambrosia) is a unique fermented bee product that mainly includes pollen, honey, and secretions of bees' salivary glands **(Vásquez and Olofsson, 2009; Barajas, Cortes-Rodriguez and Rodríguez-Sandoval, 2012)**. It is the result of lactic fermentation of pollen, collected by bees from flowers of melliferous plants and mixed by their digestive enzymes, then they are carried into the hive and kept with a thin layer of honey and bee wax. Bee bread is the main food in the hive, especially for larvae and young bees that produce royal jelly **(Kieliszek et al., 2018)**. Bee bread (BB) represents a richer source of high nutritional and functional compounds for human and honeybees than fresh pollen **(Markiewicz-Żukowska et al., 2013; Podrižnik and Božič, 2015; Denisow and Denisow–Pietrzyk, 2016; Sobral et al., 2017; Kieliszek et al., 2018)**. Compared to fresh pollen, it is characterized by a lower amount of complex polysaccharides, a shift in amino acids, proteins and lipids profiles, and an increase of simple carbohydrates and titratable acidity **(Human and Nicolson, 2006; Anđelković et al., 2012; Lee et al., 2015)**. BB is also characterized by a higher nutritional value, better digestibility, and richer chemical composition than pollen **(Habryka, Kruczek and Drygas, 2016)**. The BB results into a stable food, due to the high concentration of simple sugars (35 – 61% dry weight), low pH (3.8 – 4.3), and the presence of antimicrobial compounds **(Vásquez and Olofsson, 2009; Anderson et al., 2014; Podrižnik and Božič, 2015)**. Bee bread is the source of protein, fats, and vitamins. Although the composition of bee pollen and bee bread are similar, there are some differences. Bee bread contains less protein than bee pollen, but bee bread proteins are easier to digest **(Saa-Otero, Díaz-Losada and Fernández-Gómez, 2000; Bogdanov, 2011)**. **Nagai et al. (2004)** stated that BB contains approximately 20% protein, 3% lipids, 24 – 35% carbohydrates, 3% minerals, and vitamins. Fully balanced proteins contain all of the necessary amino acids, vitamins (C, B_1, B_2, E, K, biotin, nicotinic and folic acid), pantothenic acid, pigments, and other biologically active compounds, such as polyphenols (phenolic acid and flavonoids), carotenoids, sterols. Furthermore, enzymes (saccharase, amylase, phosphatases), are also present. In addition, BB contains more than 25 different micro- and macro- elements, such as Fe, Ca, P, K, Cu, Zn, Se, and Mg. The potential application of bee bread, as food and a nutraceutical supplement, greatly depends on its chemical composition, which varies directly with the flora of the region and the time of collection by the bees **(Markiewicz-Żukowska et al., 2013; Čeksterytė et al., 2016; Sobral et al., 2017)**. The activity of pollen (the number of vitamins and enzymes) decreases after 2 or 3 months of storage. Bee bread keeps its activity longer **(Bogdanov, 2011)**. Biologically active substances present in BB are associated with several medicinal benefits. BB has hepatoprotective, immuno-modulating, antiradiation, and adaptogenic properties **(Berene, Daberte and Siksna, 2014; Bogdanov, 2015)**. BB helps to regulate lipid metabolism and has also a positive effect on the cardiovascular system **(Nagai et al., 2004; Baltrušaitytė, Venskutonis and Čeksterytė, 2007; Tomás et al., 2017)**. BB has shown to possess *in vitro* antibacterial **(Baltrušaitytė, Venskutonis and Čeksterytė, 2007; Zerdani et al., 2011)**, antioxidant **(Zuluaga, Serrato and Quicazan, 2015; Tomás et al., 2017) and antitumor (Markiewicz-Żukowska et al., 2013; Sobral et al., 2017)** proprieties.

The aim was the evaluation of meat performance of Japanese quails after the addition of bee bread powder (perga) into their diet.

Scientific hypothesis

We expect a significant effect of bee bread on the meat performance of Japanese quails, especially on the valuable meat parts such as breast and thigh muscles.

MATERIAL AND METHODOLOGY

Animals and experimental design

Animals and diet

The experiment was carried out in the test poultry station at the Research Institute of Animal Production in Nitra. A total of 80 Japanese quails were included in the experiment. The quails were divided into four groups (10 males and 10 females in each group), as follows: the control group received no additives (C), the experimental group E1 received bee bread powder at a dose of 2 g per 1 kg of feed mixture, experimental group E2 received 4 g of bee bread powder per 1 kg of feed mixture and E3 group 6 g bee bread powder per 1 kg of feed mixture. Bee bread was of Slovak origin (Medula Ltd., Bratislava). The groups were kept under the same conditions. The quails were reared using a cage technology, each cage was equipped with a feed disperser and water intake was ensured ad libitum through a self-feed pump up to 56 days of age.

Table 1 shows the list of the ingredients and nutrient content of the basal diets (HYD-07, HYD-11), formulated to provide the nutrient requirements of quails according to the recommended reference levels. The feed mixture was produced without any antibiotics and coccidiostats.

Slaughter and measurements

At the end of the 56-day feeding period, twenty quails from each group (10 males, 10 females) were weighed and slaughtered at the slaughterhouse of the Slovak University of Agriculture in Nitra. After evisceration, the carcasses were kept at approximately 18 °C for 1 h *post mortem*. After that, the carcasses were weighed and stored at 4 °C until 24 h *post mortem*. All the weighting measurements were performed using the precision balance Kern 440 (Kern & Sohn, Germany) with an accuracy of 0.01 g. The carcass yield was calculated by dividing carcass weight with giblets and abdominal fat weight by live body weight.

Statistical analysis

The data were analyzed using the ANOVA Processed with SAS software (version 9.3, by application Enterprise Guide 4.2). Mean values and standard deviation (SD) are reported in tables. Differences between treatments were tested for significance. The level of significance was established at $p \leq 0.05$.

RESULTS AND DISCUSSION

The results of the meat performance of quails without (control group) and after application of bee bread powder (experimental groups) in their nutrition are shown in Table 2. The main indicator of the quality of poultry meat is the category of a carcass, which is determined by its nutritional status **(Maiorano and Bednarczyk, 2013)**. Generally, in quail boneless meat yields is about 77% of carcass weight, the breast muscle represents 50% of the total carcass meat yield, while leg muscle contributes about 30% **(Shanaway, 1994)**. In the case of broiler chicken, the content of muscle tissue of the carcass varies between 40% and 70% **(Maiorano and Bednarczyk, 2013)**. Generally, due to the economic reasons, broiler quails are slaughtered at approx. 5 – 6 weeks of age **(Genchev et al., 2008)**. Under the good condition of feeding and environmental conditions, the body weight gain of quails increases till the 4th week, then starts decreasing **(Shanaway, 1994; Seker et al., 2007)**.

We can conclude that application of bee bread powder in the diet of Japanese quails without gender difference did not have a significant effect on the achieved live weight except for the experimental group E2 with the addition of 2 $g.kg^{-1}$, where were achieved negative significant differences (- 20.35 g) compared to the control group.

The similar results of live weight were achieved in Japanese quails by sex, where male and female in group E2 achieved significantly the lowest live weight (male 170.32 g and female 185.06 g) among the experimental groups. The carcass yield in Japanese quail ranges from 60 to 70 –75% depending on slaughtering age, line, and sex **(Maiorano et al., 2009; Alkan et al., 2013)**. The effect of sex on slaughtering and carcass characteristics are well known in quail, and was reported as highly significant **(Khaldari et al., 2010; Narinc et al., 2010)**. The tendency of the carcass weight of Japanese quails was similar to that in live weight. Carcass yield, except for the experimental group E3 (68.89%), was at least 70% in all other experimental groups and no significant differences in this indicator were found between groups ($p \geq 0.05$). The highest carcass yield without sex difference was in the E2 group (71.84%). Considering sex, in male the highest carcass yield was in the control group (73.40%) and the lowest in the E3 group (71.70%). In female, the carcass yield was the highest in group E2 (70.73%) and again the lowest in the E3 group (66.08%).

For the sexual dimorphism, females are heavier than males, but the latter are characterized by higher carcass yield **(Marks, 1993)**. Despite that Japanese quail is not a species with a high slaughter yield, the percentage of edible meat is high. It was reported that breasts represent a considerable part of the carcass in Japanese quail **(Vali et al., 2005; Khaldari et al., 2010)** and this is a clear advantage because breast meat is favorable among consumers. The incidence on the carcass of breast muscle is ranging from 25 to 36% and for legs is ranging from 16 to 22% in Japanese quail of different ages **(Genchev et al., 2008; Alkan et al., 2013)**. An important indicator of meat performance is also the weight of valuable parts (breasts, thighs), which was significantly ($p \geq 0.05$) smaller in all experimental groups without sex difference, except for the E1 group with breast weight (52.87) versus the control group (52.57 g).

Table 1 Composition of basal diet and nutrient content of feed mixtures HYD-07 and HYD-11 per kg of diet.

Ingredients (%)	Starter feed mixture (HYD-07) (1st to 21st day)	Finisher feed mixture (HYD-11) (22nd to 56th day)
Wheat	13	15
Maize	34.8	32
Soybean meal (48% CP)	23	19.2
Fish meal (71% CP)	5	3
Malt flower	2	3
Rapeseed meal	5	7
Sunflower meal	5	4.5
Monocalcium phosphate	1	1
Fodder salt	0.2	0.3
Animal fat Bergafat	5	4
Calcium carbonate	5	10
Premix Euromix[1]	1	1
Crude protein	245	200
Fibre	50	60
Ash	140	160
Ca	8	35
P	6.5	5
Na	0.9	1.6
Lysine	14.1	11
Methionine + Cysteine	9.5	7.9
Linolic acid	10	10
ME_N ($MJ.kg^{-1}$)	12.1	11.7

Notes: CP = crude protein; Ca = calcium; P = phosphorus; Na = natrium; ME_N = nitrogen-corrected metabolizable energy; MJ = megajoule; [1]active substances per kilogram of premix: vitamin A 15 000 IU; vitamin E 20 mg; vitamin D3 2 000 IU; riboflavin 6 mg; cobalamin 20 μg; Mn 60 mg; Zn 40 mg; Fe 40 mg; Cu 6 mg; I 1 mg; Se 0.2 mg.

Based on evaluating the weight of the breast muscle regarding to the sex, we can conclude that males gained the highest value in the control group (51.81 g) and the lowest in the group E2 (46.73 g).

On the other hand, females gained the highest value in the E1 group (54.46 g) and lowest in the E2 group (47.42 g). Without sex differences, the highest values of thigh weight of Japanese quail were found in the control group (30.41 g) and the lowest in the E2 group (28.74 g). Regarding to sex, thigh weight was again the highest in the control group (29.92 g) and the lowest in the E2 group (28.49 g). In females, the thigh weight was the highest in E1 group (31.28 g) and the lowest in E2 group (28.98 g).

Based on the overall evaluation of the individual groups of experiments in the achieved meat yield of Japanese quails, the worst was the E2 group with the addition of 4 g bee bread powder per 1 kg of feed mixture.

There are no relevant researches on meat performance characteristics of Japanese quails with the addition of bee bread into their diet. However, this quail is widely used for other researches and therefore other natural supplements were tested in their diet.

Denli et al. (2005) reported a higher carcass weight (+8.2%), without significantly better carcass yield after propolis supplementation. **Canogullari et al. (2009)** reported a better weight gain after an average of 1% propolis supplementation into Japanese quail's diet. They also reported that live weight (246.3 g), carcass weight (181.7 g), carcass yield (73.7 g), liver yield (4.91 g), heart yield (2.18 g) and gizzard yield (5.45 g) were not significantly affected by selected supplementation. **Canogullari et al. (2009)** also observed pollen supplementation into quail´s diet in an amount from 5 to 20 g per kg of feed. In comparison with propolis, they found a similar weight gain, worse live weight (237.5 g) and carcass weight (177.3 g), but better carcass yield. The yield of mentioned three giblets was also higher (5.58 g, 2.31 g and 5.58 g, respectively). **Silici et al. (2007)** reported that propolis had no detrimental effect on the health but did not improve the performance parameters of quail in the first 35 days of age.

In comparison to broiler chickens, **Haščík et al. (2012)** revealed that the use of 400 $mg.kg^{-1}$ of bee pollen as a dietary supplement in broilers led to an increase in the live body weight, carcass weight, giblets weight and carcass yield in males, but it had a negative impact on females, as it decreased the body weight of the hens. **Haščík et al. (2016c)** used 400 mg of propolis extract per 1 kg of feed mixture in broiler chickens' diet. Compared with the control group (control – experimental group), they found higher live weight (2270.20 – 2316.90 g), carcass weight (1629.80 – 1669.10 g), a similar carcass yield (78.54 – 78.31%), a higher giblet weight (152.08 – 155.64 g), a similar weight of liver, gizzard and heart, respectively (40.91 – 40.61; 26.00 – 25.09 and 10.72 – 10.88 g).

Similarly to the present findings, **Haščík et al. (2014)** demonstrated that propolis extract supplementation (200, 300, 400 $mg.kg^{-1}$) increased the body weight of broiler chickens (2354.6 – 2382.9 g) in comparison with 2272.89 g in the control group. Slightly increased ($p \leq 0.05$) when the chickens were fed with the combination of humic acid with garlic powder (E2; 1.97 $g.100g^{-1}$ resp. 1.02 $g.100g^{-1}$) and humic acid plus oregano leaf powder (E3; 2.02 $g.100g^{-1}$, resp. 1.05 $g.100g^{-1}$). The content of mentioned AAs has decreased ($p \leq 0.05$) after the addition of humic acids (E1; 1.81 $g.100g^{-1}$, resp. 0.94 $g.100g^{-1}$) in comparison with the control group.

Except for bee products, other natural supplements were tested in Japanese quails' diet. Dietary supplementation with thyme, in the form of essential oil, did not lead to any significant improvement of carcass weight or carcass yield **(Denli et al., 2004; Sengül et al., 2008),** but on the other hand, the newer research carried out by **Khaksar et al. (2012)** shows a significant improvement in live body weight, carcass yield and even breasts yield**. Chantiratikul et al. (2010)** figured out that duckweed may affect carcass yield (76.7%) of Japanese quail, though not significantly.

Ghazaghi et al. (2014) claimed that peppermint *Mentha spicata* can significantly decrease feed intake without negative effects on carcass, breast and leg yields. But unfortunately, dietary supplementation with peppermint *Mentha piperita* significantly increased feed intake with a decrease of breast and leg yields **(Mehri et al., 2015)**. Green tea is known for its content of bioflavonoids, catechin and epicatechin and was tested by several authors as a dietary supplement in broiler chickens' diet **(Haščík et al., 2016b)**. However, in Japanese quail, it did not improve neither carcass (66.4%), nor giblet yields **(Kara et al., 2016)**. Comparing with our study, a canola-based diet led to a higher carcass weight (133.0 g) similar heart (1.7 g) and lower liver and gizzard weight (4.2 and 3.1 g) **(Mnisi and Mlambo, 2018)**. Both cinnamon essential oil and powder supplementations (100 $mg.kg^{-1}$ and 2 $g.kg^{-1}$ of feed) increased a live weight of Japanese quails **(Mehdipour, Afsharmanesh and Sami, 2013)**. Live body weight and carcass yield were significantly increased after the addition of a chickpea into the Japanese quail diet **(Obregón et al., 2012)**, while in our study this was not observed. Also, earthworm's powder can significantly improve the carcass yield of Japanese quail **(Morón-Fuenmayor et al., 2008; Díaz-Cuellar et al., 2009)**. **Partovi and Seifi (2018)** claimed, that in comparison with the control group, diet supplementation with *E. purpurea* extract at all concentrations decreased total feed intake ($p = 0.0017$) and that there wasn't a significant difference between experimental groups. Diet supplementation with *E. purpurea* extract decreased dressing percentage and the difference was significant between the control group with 0.025% and 0.05% groups. Diet supplementation at 0.2% caused a significant increase in dressing percentage in comparison to 0.025% and 0.05% of *E. purpurea* extract groups, yet the dressing percentage did not reach that of a control group ($p < 0.05$).

Research of **Sahin et al., (2003)** showed that dietary supplementation with vitamin C and folic acid is not suitable for Japanese quails because it led to a decrease of live body weight, carcass weight and carcass yield. Dietary L - carnitine supplementation (30, 40 and 50 $mg.kg^{-1}$) led to a decrease of live body weight (185.83 – 194.44 g) and also carcass weight (119.32 – 121.92 g), but caused an increase of giblets like liver (~2.60 g), heart (~0.92 g) and gizzard (~2.00 g) in comparison to the control group **(Sarica et al., 2005)**.

Table 2 Effect of bee bread powder on meat performance parameters of quails.

Parameter	Sex	C	E1	E2	E3	*p*-value
Live body weight (g)	Male	184.26 ±7.03^{a}	183.58 ±4.80^{a}	170.32 ±4.88^{b}	184.80 ±3.93^{a}	0.0335
	Female	211.82 ±10.84^{a}	209.30 ±3.12^{a}	185.06 ±7.30^{b}	208.38 ±5.82^{a}	0.0335
	♂+♀	198.04 ±16.89^{a}	196.44 ±14.08^{a}	177.69 ±9.73^{b}	196.59 ±13.28^{a}	0.0147
Carcass weight (g)	Male	122.04 ±6.33	121.44 ±4.38	113.11 ±5.43	120.56 ±2.49	0.0663
	Female	128.13 ±9.50^{ac}	129.62 ±10.91^{a}	116.50 ±3.96^{bc}	123.42 ±9.69^{ab}	0.0472
	♂+♀	125.08 ±8.26^{a}	125.53 ±8.95^{a}	114.81 ±4.82^{b}	121.99 ±6.84^{a}	0.0054
Giblets weight (g)	Male	13.22 ±0.68^{a}	13.12 ±0.36^{a}	11.17 ±0.65^{b}	11.90 ±0.63^{a}	0.0335
	Female	14.70 ±1.08	13.58 ±1.69	14.30 ±1.51	14.36 ±0.85	0.2417
	♂+♀	13.96 ±1.15	13.35 ±1.18	12.74 ±1.98	12.74 ±1.98	0.1670
Carcass yield (%)	Male	73.40 ±0.97	73.30 ±1.15	72.95 ±1.24	71.70 ±1.85	0.1290
	Female	67.39 ±1.80	68.40 ±5.43	70.73 ±2.48	66.08 ±3.72	0.0928
	♂+♀	70.40 ±3.45	70.85 ±4.51	71.84 ±2.19	68.89 ±4.05	0.0917
Liver (g)	Male	4.20 ±0.43^{ac}	4.28 ±0.28^{a}	3.38 ±0.45^{bc}	3.53 ±0.41^{bc}	0.0335
	Female	5.61 ±0.82	4.70 ±0.55	5.68 ±0.94	5.56 ±0.71	0.1280
	♂+♀	1.79 ±0.25	4.49 ±0.47	4.53 ±1.40	4.54 ±1.19	0.4370
Gizzard (g)	Male	3.57 ±0.27^{a}	3.75 ±0.47^{a}	2.88 ±0.42^{b}	3.13 ±0.57^{ab}	0.0335
	Female	3.88 ±0.29	3.76 ±0.53	3.32 ±0.52	3.55 ±0.30	0.1290
	♂+♀	3.73 ±0.31^{a}	3.76 ±0.47^{a}	3.10 ±0.50^{b}	3.34 ±0.48^{ab}	0.0106
Heart (g)	Male	1.79 ±0.26	1.74 ±0.23	1.62 ±0.21	1.71 ±0.10	0.5452
	Female	1.78 ±0.27	1.71 ±0.26	1.61 ±0.21	1.61 ±0.24	0.5464
	♂+♀	1.79 ±0.25	1.72 ±0.23	1.61 ±0.20	1.66 ±0.19	0.3027
Neck (g)	Male	3.66 ±0.26	3.35 ±0.24	3.29 ±0.34	3.54 ±0.41	0.0928
	Female	3.43 ±0.40	3.40 ±0.56	3.70 ±0.48	3.64 ±0.59	0.3704
	♂+♀	3.54 ±0.34	3.38 ±0.41	3.50 ±0.45	3.59 ±0.48	0.2556
Breast (g)	Male	51.81 ±2.70	51.28 ±3.71	46.73 ±2.21	49.85 ±2.17	0.0663
	Female	53.32 ±3.65	54.46 ±5.38	47.42 ±4.03	51.03 ±4.92	0.0928
	♂+♀	52.57 ±3.13^{a}	52.87 ±4.67^{a}	47.08 ±3.08^{b}	50.44 ±3.64^{ab}	0.0076
Thigh (g)	Male	29.92 ±1.88	29.14 ±0.87	28.49 ±2.28	29.90 ±1.20	0.3235
	Female	30.89 ±3.02	31.28 ±2.89	28.98 ±1.29	30.48 ±3.16	0.4250
	♂+♀	30.41 ±2.43	30.21 ±2.31	28.74 ±1.76	30.19 ±2.27	0.2274

Notes: Values are shown as mean ± *SD* (standard deviation); C = control group; E1, E2, E3 = experimental groups; a, b, c = means within a row with different superscripts differ significantly at $p \leq 0.05$, one-way ANOVA.

Raji et al. (2015) observed much lower meat performance characteristics of Japanese quails in comparison with our research. They examined live weight, carcass weight, carcass yields and the weight of the breast and thigh muscle, according to their sex, color type, weight group, and age.

The average live weight was 130.56 g (ranging from 97.19 to 162.67 g); carcass weight of 91.65 g (ranging from 67.60 to 119.54); carcass yield 70.24% (ranging from 68.02 to 72.17); breast muscle weight 27.48 g (ranging from 15.76 to 39.32 g); and thigh muscle weight 19.89 (ranging from 12.61 to 29.30 g).

Some of our results were compared with the results of **Lember and Laan (2012)** who compared male and female carcass characteristics of Estonian, Pharaoh and French White quails. For example, the live body and carcass weight of Estonian quail respectively were similar to our results (comparison of males – females): live body weight 184.4 – 208.6 g and carcass weight 119.1 – 128.8 g. According to the control slaughter, the heaviest quails at the age of 42 days were French white quails (261.2 – 302.4 g), their carcasses were also the biggest (169.8 – 185.8). Females of all quail strains in this study had a bigger live weight at the age of 6 weeks. The lowest weights of the breast and thigh muscle were in Estonian quail male (37.4 and 24.4 g), while the highest in French White (63.0 and 38.5 g). **Nasr et al. (2017)** observed performance, carcass traits, meat quality and amino acid profile of different Japanese quails' strains. There was no significant difference among the quail chicks body weight of different plumages colour at 1 day of age. While at the 6th week of age, the white quail had the highest body weight (205.16 g) and the brown quail had the lowest body weight (174.68 g). The white quail had the highest weight of slaughter and carcass, dressing percentage, carcass yield, weight of liver, gizzard, heart and spleen (197.27 g, 169.27 g, 91%, 82%, 6.63 g, 6.53 g, 2.27 g and 0.40 g, respectively) when compared with the other plumage colours.

CONCLUSION

Based on the results of the experiment, we can conclude that the application of 2, 4 and 6 mg bee bread powderon 1 kg the feed mixture in the nutrition of Japanese quails did not have a positive effect on their achieved meat performance. We propose not to apply this preparation in the amount we tested in their nutrition, respectively to look for other alternative solutions for its application in quails nutrition, alternatively others poultry species.

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Compliance with ethical standards

Birds care, manipulation and handling complied with the regulations of the European Parliament and the European Council Directive on the protection of animals used for scientific purposes (2010/63/EU). The research Animal Ethic Committee of Research Institution approved this experiment.

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FACTORS AFFECTING RAW MILK QUALITY OF DAIRY COWS UNDER PRACTICAL CONDITIONS

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ABSTRACT
Under the practical conditions, it is important to evaluate the factors affecting milk performance. Data from test day yield and milk components should be useful for such evaluation. The aim of the experiment was to study the effect of season, udder health (by somatic cell counts SCC), parity, stage of lactation on milk production, milk components, and SCC under the practical conditions. Also, the frequency of incidence of high SCC during the season was observed. The experiment was realized on one dairy farm in dairy practice. The experiment lasted from December 2015 to October 2017. We examined 481 Holstein dairy cows (6910 milk samples). Milk samples were collected once per month – performed by recording test day. Only cows with 9 – 11 test days were evaluated. The effect of season, parity, stage of lactation, and SCC influenced most of the studied traits. The milk yield was highest at 2nd lactation. In the following lactations, the milk yields were decreasing. The SCC significantly increased with advanced parity. The elevated SCC was found in the beginning and in the final part of lactation. SCC as a factor significantly reduced milk yield, lactose content but increased fat and protein content. In conclusion, under practical conditions, the management should use the data from test days and analyze them for a better understanding of the performance efficiency at the farm level and for implementing more sophisticated decision making in farming.

Keywords: dairy cows; data; milk; performance

INTRODUCTION
Raw milk production and its quality at the farm level depends on many factors of external and internal conditions. Most often there are described external factors especially heat stress, season, humidity **(Lambertz, Sanker and Gauly, 2014)**, and internal factors like parity, stage of lactation, udder health, metabolic status **(Tančin, Ipema and Hogewerf, 2007; Tančin et al., 2007; Penev et al., 2014)**. Most of the articles published the results under experimental conditions, but conditions at the farm level are often different due to management practices, even if the breeding conditions are the same. Thus, the effect of above-mentioned factors under practical conditions could be an important source of information for the optimal management of each dairy farm.

One of the most important information coming from the dairy practice is data obtained from milk recording test days (milk yield, milk components, somatic cell count – SCC) performed in a monthly period. After processing, these data could be used for the management of dairy cows. One of the most important information coming from test day is data indicating udder health through SCC **(Tančin, 2013)**. SCC is a gold standard in diagnosing of any forms of mastitis of the udder **(Pyörärlä, 2003; Bobbo et al., 2017)** and it is also negatively related to milk yield and its components **(Barkema et al., 1999, Kull et al., 2019)**, and to technological quality of milk **(Santos, Ma and Barbano, 2003; Leitner et al., 2004, Franzoi et al., 2020)**. SCC data are used in the selection of dairy cows **(Strapakova, Candrak and Strapak, 2016)**. Thus, the economic impact of mastitis should be seriously considered **(Petrovski, Trajcev and Buneski, 2006)** at the farm level. SCC in raw milk is also affected by other internal and external factors like stage of lactation, parity **(Tančin, Ipema and Hogewerf, 2007; Tančin et al., 2007)**, frequency of milking **(Hogeveen et al., 2001)**. However, from the health point of udder, these factors did not increase SCC dramatically as the mastitis do.

Milk production, its components, and udder health (represented by SCC) are data available for dairy farms through regular milk recording test day. However, at the farm level, these data are not processed correctly or even not used. Moreover, critical scientific evaluation of these data could be also important. The aim of the experiment was to study the effect of season, parity, stage of lactation on milk production, its components, and SCC under the practical conditions.

Scientific hypothesis

The season, udder health, parity, stage of lactation significantly influence milk yield, milk components and SCC. SCC reduced milk yield and changed milk components.

MATERIAL AND METHODOLOGY

The experiment was realized in one dairy farm in dairy practice. There were black Holstein cows on the farm with average year milk production 10,500 kg. The experiment lasted from December 2015 till October 2017, during which we examined 481 dairy cows. In total, 739 records of cows were evaluated, as in some cows also 2nd, 3rd, and higher lactations were included. Thus, 36% of evaluated cows were on their first lactation, 26% on their second, 18% on third, 12% on fourth, and 8% on the fifth lactation.

The dairy cows were housed in a free housing system with cubicles. Animals were milked three times a day in 2x10 parallel milking parlour. The parlour was equipped with automatic devices for cluster removal. The milking routine included also udder cleaning with towel and fore-stripping. The cows were fed by a total mix ration two times a day.

Milk samples were collected once per month – on the official recording test day. Only cows with 9 – 11 test days were involved in the statistical examination of data. Some samples were excluded from the evaluation due to insufficient milk collection which was insufficient for analysis of all milk parameters. Thus, in total 6910 samples from the experimental period were included and used for statistical evaluation. The basic milk components (fat, protein, lactose) were determined by MilkoScan FT120 (Foss, Hillerød, Denmark) and somatic cells count were determined using a Fossomatic 90 (Foss Electric, Hillerød, Denmark) after heat samples at 40 °C for 15 min.

Animals on the basis of SCC were divided into four groups: low (SCC $<3x10^5$ cells.mL^{-1}), middle (SCC between $3x10^5$ and $6x10^5$ cells.mL^{-1}), high (SCC between $6x10^5$ and 10^6 cells.mL^{-1}) and the highest (SCC $>10^6$ cells.mL^{-1}). We also created 5 groups of animals according to their parity numbers (first, second, third, fourth, fifth, and subsequent lactation). The stage of lactation was divided into four groups (into intervals of approximately 90 days) – in the first group the cows were on their 52.91 ±21.76 days of lactation, in the second one on 135.01 ±25.96 days, in the third on 224.58 ±26.22 days and the fourth on 296.85 ±16.72 days of lactation. In terms of the season we considered four groups – winter (December, January, February), spring (March, April, June), Summer (June, July, August), Autumn (September, October, November).

Statistical analysis

Obtained data were processed by Microsoft Excel and statistically evaluated by SAS/8.2 (2002). The model was tested by using Fisher's F-test. Differences between the levels of the effects were tested by Scheffe multiple range test for studied traits. Data are presented as LSmeans ± standard error for evaluation of somatic cells the following model was used:

$$y = X\beta + Zu + e$$

Where:

y –was the measurements for somatic cell counts; β – the fixed effects of parity, stage of lactation, season, SCC group; u – random effect of cow, u ~ N (0, I δ2c); e – random error, assuming e ~ N (0, I δ2e); X, Z – incidence matrices for fixed effects and random cow effect, resp.

RESULTS AND DISCUSSION

There is the basic statistics of evaluated data in Table 1. The mean of the daily milk production corresponded to the data obtained from the well managed farm **(Tančin et al., 2006)**. In this herd, the mean of SCC was above the limit for SCC in bulk milk tank **(EU regulation 853/2004)**. On average 76.66% of samples were in the low SCC group and 10.25% of samples were in the highest SCC group of the studied herd. Some seasonal effect was also observed, were in the low SCC group the lowest percentage (70.55%) of samples were in summer 2015 and the highest percentage of samples (81.65 %) were in autumn 2016 (Figure 1).

The effect of season, parity, stage of lactation, and SCC groups influenced most of the studied traits (Table 2). The effect of SCC on fat/protein ratio and parity on protein in milk was not found. The highest LSmeans of milk yield was detected in summer 2015 (32.79 ±0.45 kg) and the lowest in autumn 2016 (27.04 ±0.38 kg, p <0.05). Throughout the study, there were higher milk yields in Spring and Summer with the following decrease in Autumn periods. The highest fat content was found in Winter 2015/2016 (4.41 ±0.03%) and the lowest in Summer 2017 (3.76 ±0.04%, p <0.05). The concentration of protein was the highest in Winter 2016/2017 (3.39 ±0.02%) and the lowest in Summer 2015 (3.11 ±0.02%, p <0.05). The range of lactose LSmeans were from 4.66 ±0.01 % (Autumn 2015) to 4.78 ±0.01% (Winter 2014/15 and Spring 2015). The SCC was lowest in Spring and Summer 2016 (5.12 ±0.03 logPSB.mL^{-1}) and the highest in Spring and Summer 2015 and Summer and Autumn 2017 (5.24 ±0.03 logPSB.mL^{-1}). The significant differences among seasons in SCC were only between summer 2015 and summer 2016 (p <0.05). Though the seasons of the year significantly influenced studied trials in dairy cows, in general, the same seasons did not have a similar effect on studied traits (Table 3). The significant differences of LSMeans at fixed factor „Season" can be found in Table 4.

Most frequently in the science, the season is discussed in relation to SCC. Summer period seems to be a risk factor for udder health in the fact that environmental pathogens caused a higher incidence of mastitis during the summer period **(Smith, Todhunter and Schoenberger, 1985; Penev et al., 2014)** as a possible consequence of suitable living conditions for bacteria **(Mallet et al., 2012)**. This was also confirmed in our previous work in dairy practice **(Tančin 2013)**, where there was a significant increase of SCC during the period of May, June, and July as compared with winter months of the year. But at present work, the summer was not confirmed as the most critical season (Table 3). Another work showed more critical period on mastitis occurrence in the winter season **(Olde Riekerink, Barkema and Stryhn, 2007)**. Under the conditions of healthy mammary glands, the season was pointed out to have no significant influence on SCC **(Malinowski, 2001)**.

In our study, we had summers with the highest, but also the lowest SCC, so other important factors like management and effective mastitis control program might be more important. The climatic, microclimatic conditions and feeding vary from year to year that could diminish or extend the difference among seasons. The stage of lactation is an important factor affecting milk performance. Milk yield and protein content significantly decreased from the first to the fourth stage of lactation (Table 5). Fat content was the highest in the fourth stage and lactose was reduced during the last stages of lactation (third and fourth stage). The SCC significantly changed from the stage to stage of lactation with higher SCC at the first stage, with a reduction in second and again with an increase in the third and the fourth stage of lactation (Table 5). The changes of SCC during lactation showed the most critical period for SCC in the beginning and in the end of lactation. These changes are generally known **(Tančin, 2013)** and again were confirmed at practical conditions. A significant effect of the stage of lactation in dairy cows was also documented by **Laevens et al. (1997)** and **Sebastino, Uribe and Gonzalez (2020)**. From the management point of view, the period early postpartum and before drying are critical for udder health. Therefore, more attention should be focused on the care of cows during both mentioned periods of lactation.

The milk yield and its components and SCC in relation to the parity are shown in Table 5. Milk yield was the highest at second lactation with decreasing in the following lactations. The SCC significantly increased with advanced numbers of lactation. Especially the group of cows on their fifth and subsequent lactations had 5.59 ±0.05 logx.mL^{-1}, compared to primiparous cows with 4.88±0.03 logx.mL^{-1}. There were no significant differences between the first two groups of parities indicating a relatively low increase of health problems during the first two lactations. In our earlier study **(Tančin, Ipema and Hogewerf, 2007)** the multiparous cows had only numerically higher SCC as

Table 1 The basic statistics of evaluated data.

Parameter	N	Minimum	Maximum	Mean	Std Error
Yield, kg	6910	2.50	56.80	33.07	0.11
Fat, (F) %	6910	1.18	9.90	3.88	0.01
Protein, (P) %	6910	2.02	9.33	3.16	0.004
Lactose, %	6910	2.62	5.42	4.83	0.002
SCC, x.10^3 mL^{-1}	6910	4.00	29603	503.59	18.98
logSCC	6910	3.30	7.47	5.02	0.01
Ration F/P	6910	0.32	3.05	1.23	0.002

Table 2 Results of variance analysis for milk yield and milk component traits (statistical significance of the Sheffe-test).

Factors/Traits	Yield (kg)	Fat (%)	Protein (%)	Lactose (%)	Ratio F/P	logSCC
Season	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Lactation stage	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Parity	<.0001	<.0001	0.5079	<.0001	<.0001	<.0001
SCC group	<.0001	<.0001	<.0001	<.0001	0.8163	

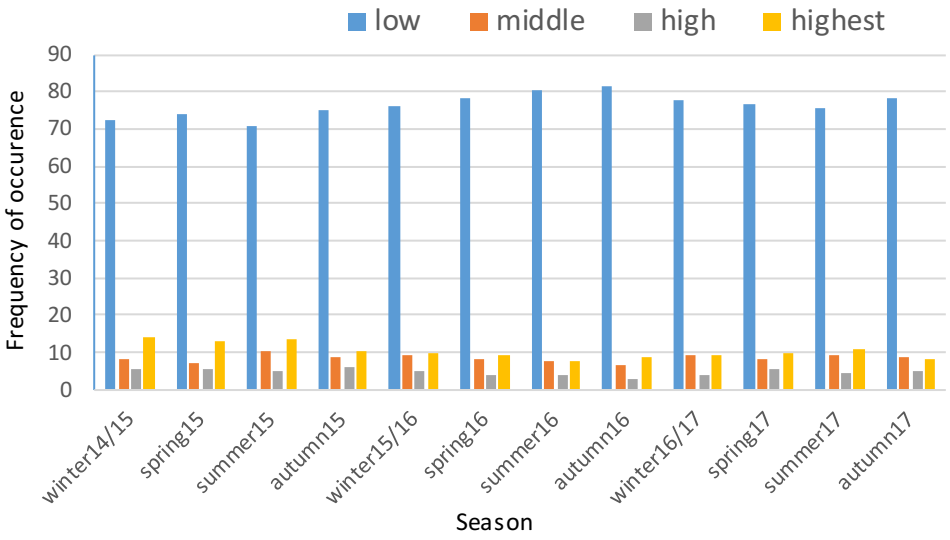

Figure 1 The effect of season and frequency of occurrence (%) of samples in SCC group (from 2014 to 2016 on occurrence of samples in different SCC group. Low (SCC <3x105 cells.mL^{-1}), middle (SCC between 3x105 and 6x105 cells.mL^{-1}), high (SCC between 6x105 and 106 cells.mL^{-1}) and highest (SCC >106 cells.mL^{-1}).

Table 3 Least squares means for milk yield and its composition traits according season.

N	Factor	Parameters											
		Yield (kg)		Fat (%)		Protein (%)		Lactose (%)		Lactose (%)		logSCC	
		LSM	Std. error	LSM	Std. error	LSM	Std. error	LSM	Std. error	LSM	Std. error	LSM	Std. error
1	Winter 2014/15	33.48	0.55	3.92	0.05	3.36	0.02	4.78	0.01	1.18	0.01	5.18	0.04
2	Spring 2015	32.63	0.49	3.92	0.04	3.24	0.02	4.78	0.01	1.21	0.01	5.24	0.04
3	Summer 2015	32.79	0.45	3.95	0.04	3.11	0.02	4.70	0.01	1.28	0.01	5.24	0.03
4	Autumn 2015	29.11	0.43	4.07	0.04	3.29	0.02	4.66	0.01	1.24	0.01	5.22	0.03
5	Winter 2015/16	30.02	0.41	4.30	0.04	3.37	0.02	4.70	0.01	1.28	0.01	5.17	0.03
6	Spring 2016	30.43	0.39	4.00	0.04	3.29	0.02	4.72	0.01	1.22	0.01	5.12	0.03
7	Summer 2016	29.49	0.39	4.00	0.04	3.20	0.02	4.72	0.01	1.26	0.01	5.12	0.03
8	Autumn 2016	27.04	0.38	4.08	0.04	3.36	0.02	4.70	0.01	1.22	0.01	5.17	0.03
9	Winter 2016/17	27.32	0.37	4.41	0.03	3.39	0.02	4.72	0.01	1.31	0.01	5.17	0.03
10	Spring 2017	30.41	0.37	3.98	0.03	3.24	0.02	4.73	0.01	1.23	0.01	5.22	0.03
11	Summer 2017	31.54	0.40	3.76	0.04	3.17	0.02	4.77	0.01	1.19	0.01	5.24	0.03
12	Autumn 2017	27.56	0.53	4.10	0.05	3.24	0.02	4.74	0.01	1.27	0.02	5.24	0.04

Table 4 Significant differences of LSMeans at fixed factor „Season“ (explanation in table 3 „N“).

Yield	Fat	Protein	Lactose	Ratio F/P	logSCC
1: 4; 5; 6; 7; 8; 9; 10; 12; 2: 4; 5; 6; 7; 8; 9; 10; 12; 3: 4; 5; 6; 7; 8; 9; 10; 12; 4: 8; 9; 11 5: 8; 9; 6: 8; 9; 12; 7: 8; 9; 11; 8: 10; 11; 9: 10; 11; 10: 12; 11: 12;	1: 5; 9; 2: 5; 9; 3: 5; 9; 4: 5; 9; 11 5: 6; 7; 8; 10; 11; 6: 9; 11; 7: 9; 11; 8: 9; 11; 9: 10; 11; 12; 10: 11; 11: 12	1: 2; 3; 7; 10; 11; 2: 3; 5; 8; 9; 3: 4; 2; 6; 7; 8; 9; 10; 12; 4: 5; 7; 9; 11; 5: 6; 7; 10; 11; 12; 6: 7; 9; 11; 7: 8; 9; 8: 9; 11; 12; 9: 10; 11; 12;	1: 3; 4; 5; 6; 7; 8; 9; 10 2: 3; 4; 5; 6; 7; 8; 9; 10; 3: 4; 11; 4: 5; 6; 7; 8; 9; 10; 11; 12; 11: 5; 6; 7; 8; 9; 10;	1: 3; 4; 5; 6; 7; 9; 10; 12; 2: 3; 5; 9; 3: 6; 8; 11; 4: 9; 5: 6; 8; 10; 11; 6: 9; 7: 9; 11; 8: 10; 9: 10; 11; 11: 12;	3: 7;

compared with primiparous cows and this difference is in agreement with other findings **(Laevens et al., 1997)**. Recently **Sebastino, Uribe and Gonzalez (2020)** showed a significant increase of SCC with parity.

SCC as an internal factor significantly reduced milk yield and lactose content on one side and increased the content of fat and protein (Table 5). A similar effect of SCC on milk yield we demonstrated earlier **(Tančin, Ipema and Hogewerf, 2007)**. However, in another study, the lowest fat, SNF, protein, and lactose were determined in milk with SCC >500x10^3 cells.mL^{-1} **(Kull et al., 2019)**. It was further observed that fat % expressed a negative phenotypic correlation with SCC **(Wagay et al., 2018)**. Recently, **Concalves et al. (2018)** also demonstrated the daily milk losses caused by increased SCC. **Bezman et al. (2015)** found out a decrease in milk yield and lactose with increased SCC caused by the presence of mastitis pathogens. In milk with high SCC, the last-mentioned authors also demonstrated an increase or decrease of the protein in milk and a decrease or no changes of fat. These changes were influenced by the presence of different pathogens. In our study, we did not have any information about the presence of pathogens.

Table 5 Least squares means milk yield and its components according estimated factors.

	Traits											
	Yield (kg)		Fat (%)		Protein (%)		Lactose (%)		Ration F/P		logSCC	
Factor	LSM	Std. error	LSM	Std. error	LSM	Std. error	LSM	Std. error	LSM	Std. error	LSM	Std. error
Stadium1	34.41^{a}	0.38	4.08^{a}	0.03	3.14^{a}	0.02	4.76^{a}	0.01	1.31^{a}	0.01	5.17^{a}	0.02
Stadium2	33.80^{a}	0.37	3.82^{b}	0.03	3.15^{a}	0.02	4.79^{a}	0.01	1.21^{b}	0.01	5.10^{b}	0.02
Stadium3	28.64^{b}	0.37	4.01^{a}	0.03	3.34^{b}	0.02	4.71^{b}	0.01	1.20^{b}	0.01	5.18^{a}	0.03
Stadium4	23.75^{c}	0.44	4.24^{c}	0.04	3.46^{c}	0.02	4.65^{c}	0.01	1.22^{b}	0.01	5.29^{c}	0.03
1st Lactation	28.85^{a}	0.44	4.12^{a}	0.03	3.25^{a}	0.02	4.76^{a}	0.01	1.27^{a}	0.01	4.88^{a}	0.03
2nd Lactation	31.53^{b}	0.39	3.94^{b}	0.04	3.28^{a}	0.02	4.76^{a}	0.01	1.20^{b}	0.01	4.93^{a}	0.02
3nd Lactation	31.27^{bc}	0.44	3.99^{ab}	0.04	3.29^{a}	0.02	4.73^{a}	0.01	1.22^{bc}	0.01	5.17^{b}	0.03
4nd Lactation	30.20^{ab}	0.51	4.13^{ac}	0.04	3.28^{a}	0.02	4.70^{b}	0.01	1.27^{ac}	0.01	5.36^{c}	0.04
Lactation ≥5	28.90^{a}	0.65	4.00^{abc}	0.04	3.26^{a}	0.03	4.67^{b}	0.01	1.23^{abc}	0.02	5.59^{d}	0.05
SCC low	33.14^{a}	0.31	3.90^{a}	0.04	3.16^{a}	0.01	4.84^{a}	0.01	1.24	0.01		
SCC middle	29.79^{b}	0.40	4.03^{b}	0.04	3.28^{b}	0.02	4.75^{b}	0.01	1.23	0.01		
SCC high	29.66^{b}	0.47	4.10^{b}	0.05	3.31^{cb}	0.02	4.71^{c}	0.01	1.24	0.01		
SCC highest	28.02^{c}	0.40	4.12^{b}	0.06	3.34^{c}	0.02	4.61^{d}	0.01	1.24	0.01		

Note: a,b,c,d – means with different letter within column and factor significantly differs at p <0.05.

CONCLUSION

At the studied farm level, the season, parity, and stage of lactation significantly influenced milk performance and SCC. Seasons showed unbalanced milk production, milk components, and also SCC throughout the study period. The SCC significantly increased with parity and was highest at the beginning and at the end of lactation. The SCC significantly reduced milk yield and lactose but increased fat and protein content in milk. Processing the data from the recording test days and their implementation at the farm level could contribute to better managing animal breeding.

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INTELLIGENT CONTROL SYSTEM FOR MINCED MEAT PRODUCTION

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ABSTRACT

This article presents the theoretical aspects of developing a control system for the processing of frozen raw meat by cutters in automatic mode. The method for analytical calculation of the productivity rate of meat cutting by a cutter with a screw tooth provides an accuracy for which relative error does not exceed 6%. The authors show automatic process control in minced meat production using a control system computer (CSC), with the aim of building an automatic control system (ACS) for chopping raw materials frozen in the form of blocks. The task of ACS synthesis was solved: the system structure and its elements were chosen, the topology of their cause-and-effect relationships and an algorithm of control devices were developed, and their parameters were determined. The ACS's control loop scheme for raw material cutting speed was realized, where an assembly of devices was chosen as the object of management (OM): the squirrel cage induction motor (SCIM) of the cutting mechanism drive; the frequency converter (FC) of the supply voltage, which changes the rotation speed of the SCIM (the rotation speed of the milling cutter); and the milling cutter of the chopper. The shaping filter method was used, to predict the size of the meat chips produced, to modulate the perturbation acting on the system from the load side. Based on the single-stage chopping of raw meat, an automatic line is created for producing meat products, with a minced meat quality management system based on artificial intelligence on the principle of 'unmanned technology'.

Keywords: automation of sausage production; innovative approach; minced meat production; machine control

INTRODUCTION

In the technology of meat and meat product processing **(Lisitsyn et al., 2004; Zajác et al., 2015; Herrero et al., 2008)**, it is noted that the structural and mechanical properties are determined largely by the particle size of the chopped raw material, i.e. the degree of chopping. It is also noted that with the help of structural and mechanical properties, it is possible to control the technological parameters of raw materials and minced meat, and the product quality at any stage of the technological process of minced meat preparation, as well as the texture of finished products.

To obtain ready meat products with specified characteristics, first of all, it is necessary to prepare minced meat under rational chopping modes. The duration of chopping (τ_k, s) depends on the type of chopping machine (its geometric and kinematic parameters) and is determined by the following equation **(Kosoj, Malyshev and Yudina, 2005)**:

$$\tau_\kappa = A_1 K_p \left[exp(0.326 K_{vd} + 0.35)\right] \Omega^{-1} \quad (1)$$

Where A_1 is a coefficient equal to 1×10^5 m^3.(kg.s)$^{-1}$; K_p is a coefficient that takes into account additional cutting of the product due to the pressure in the cutting chamber; K_{vd} is the ratio of protein and fat in the meat; and Ω is the generalizing kinematic characteristics of the chopping machine determined by the following dependence:

$$\Omega = f_0 \frac{W_n^2}{W_f}, \quad m^3 / \left(kg \cdot s^2\right) \quad (2)$$

Where: f_0 is the cutting capacity of the machine equal to $f_0 = z n_n F_d / 60 m_f$ m^2/(kg·s) in which z is the number of knives in the cutting tool; n_n is the frequency of blade rotation; F_d is the cross-sectional area of the minced meat layer made with a knife in one revolution, m^2; and m_f is the weight of minced meat, kg. W_n is the circumferential rotation speed of knives at their greatest radius r_n, equal to $W_n = 2\pi r_n n_n / 60$ m.s^{-1}; and W_f is the feed rate of minced meat to the cutting mechanism along the axis of knife rotation, m.s^{-1}.

Machine control of the single-stage chopping mode of initial frozen meat raw materials will allow rational management of the entire technological process on an automatic line developed for sausage production. In this regard, the purpose of automatic control of chopping raw materials by the rotary cutting method can be formulated

as follows: 1) it shall maintain a predetermined degree of chopping of the raw meat under the conditions of raw meat structural heterogeneity and textural features; 2) it shall calculate while chopping the forecast changes in meat particle (meat chip) size by means of an automatic control system (ACS) for the technological process. To achieve these goals, it is necessary to solve the task of ACS synthesis. For this purpose, it is necessary to choose the structure of the system, its elements, and the topology of cause-and-effect relations between them; and to develop algorithms for the control devices and values of their parameters, for example, regulator settings **(Pupkov et al., 2004b; Shaykhutdinov et al., 2016; Yao et al., 2010; Zhou and Wang, 2012; Balejko, Nowak and Balejko, 2012)**.

Frozen meat cut with cutters shows significant differences to that cut with some traditional materials (metals, wood, plastics) – significant heterogeneity of the structural and textural characteristics of frozen meat raw materials affects the quality of chopping **(Kapovsky et al., 2017; Lisitsyn et al., 2016, Lisitsyn et al., 2017; Kapovsky, Zakharov and Nikitina, 2019)**. Different amounts of ice (water) in frozen meat at different storage temperatures and the presence of fat and connective tissues are the structural signs of its heterogeneity. When cutting frozen meat blocks, the quality of chopping is affected by the different orientation of muscle fibres in the mass of the product block in relation to the cutter blades, which is a textural sign of the heterogeneity of the raw material.

The impact of influence factors on the raw materials chopping degree by the rotary cutting method is random due to the above-noted heterogeneity of raw materials. For example, when the cutting blade of the milling cutter is positioned longitudinally to the bundle of fibers in the muscle tissue, the width of the cut layer of meat will be bigger in comparison with the transverse positioning of the same fibers. The orientation of the cutting edge of the chopper working blade during the chopping in reference to individual pieces of meat in the frozen block of raw materials is determined by their random arrangement after molding and freezing in a freezer mold. Taking into account the peculiarities of the meat block topology, it is advisable to analyze statistically the size of meat chips of minced meat obtained in result of chopping experimental blocks of frozen raw materials with cutters of different designs and geometry. The data of statistical analysis will allow defining the regularities of the meat chips size of as a random process, which numerical parameters are determined by the operating parameters of the raw materials chopping (speed of chopping and rate of feed of a block of frozen meat into the chopping zone) and the used chopper blade.

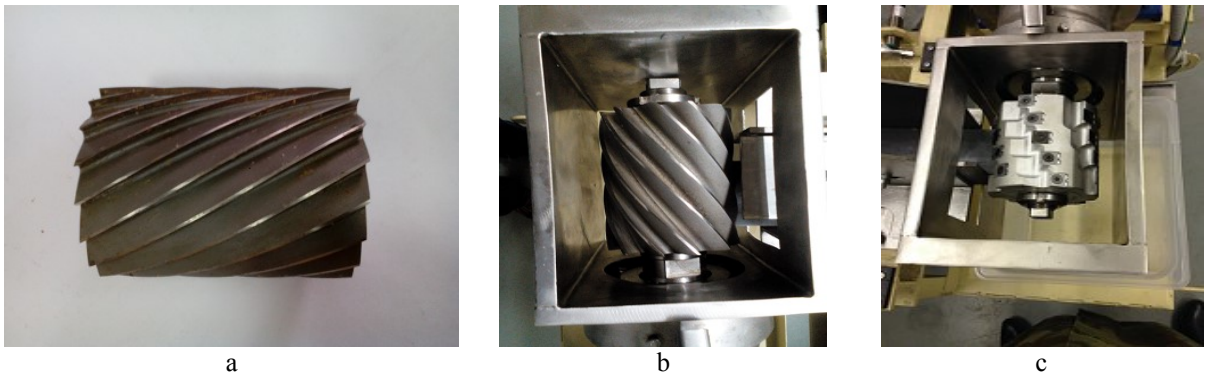
Scientific hypothesis

Let's assume the formation of the chip size as a random process, ACS shall be arranged to calculate the predicted values of the numerical parameters estimates (expected mean and variance) of the statistical distribution of the typical particle size of the chopped meat to determine the parameters of its further processing on an automated line. The specified forecast must correspond to the specified accuracy and reliability, determined by the requirements applied for the manufactured meat products. The calculation by ACS of the predicted value of the typical size of meat chips shall be based on imitating of the mathematical modeling of one-stage chopping of raw frozen meat with a multi-blade chopper tool.

MATERIAL AND METHODOLOGY

To study the single-stage process of frozen meat block cutting by cutters, the following experimental installation IBF-1 (meat block chopper with rotary cutting modification 1) was created. The unit was equipped with cutters of different designs and geometries (Figure 1).

The parameters of the cutting conditions (cutting speed of the rotary cutter and feed rate in the chopping zone) were set by the appropriate settings of the supply voltage frequency converters which work on the electric motor drives of the cutting and feeding systems of the IBF-1 installation. Experimental blocks of frozen meat preliminary cut from industrial-size blocks (second-grade beef and semi-fat pork) were chopped on the experimental installation. In accordance with **GOST R 54704 (2011)** 'Frozen blocks of trimmed meat', the mass fraction of connective and fat tissue in beef was 20% or less; in pork it ranged from 30% to 50%. The temperature of the meat in the centre of the experimental unit before chopping was -12 to -14 °C.



a b c

Figure 1 Rotary cutters, components of chopper IBF-1. Note: a – cylindrical rotary cutter according to **GOST 29092 (1991)**, type 1 (solid), version 1 (fine tooth), with an external diameter of 100 mm, inner diameter of 40 mm, length of 125 mm, and 18 teeth; b – cylindrical rotary cutter according to **GOST 29092-91**, type 1 (solid), version 2 (with large teeth), with an external diameter of 100 mm, inner diameter of 40 mm, length of 125 mm, and 12 teeth; c – rotary shaft-mounted cutting planer with TM21M EEC carbide inserts (Freud, Italy) with an external diameter of 100 mm, inner diameter of 30 mm, and length off 100 mm.

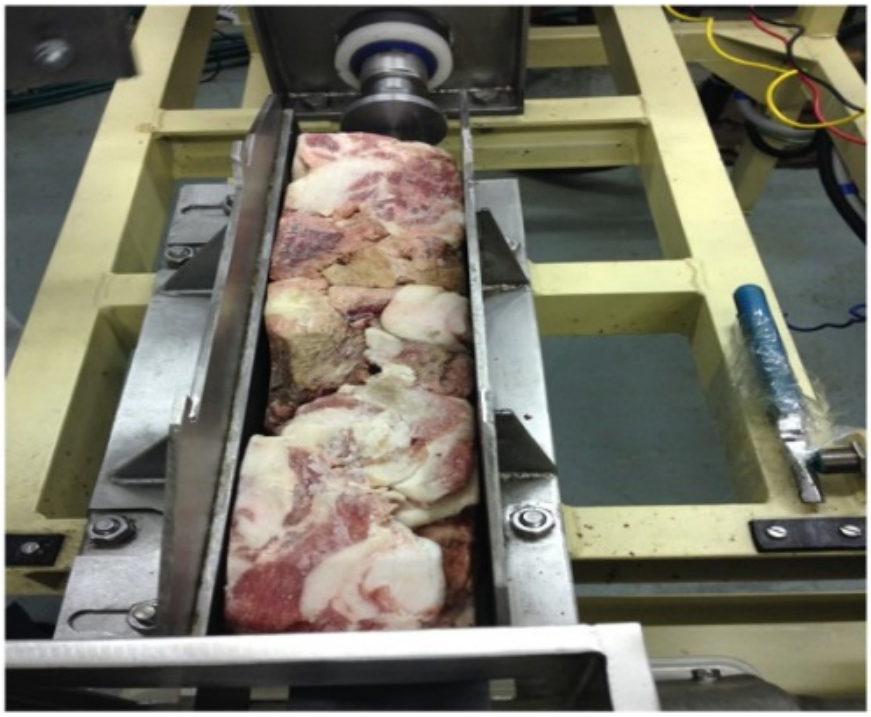


Figure 2 Experimental frozen meat block placed in the guide chute of the IBF–1 chopper.

The placement of the experimental frozen meat block in the guide chute of the IBF-1 chopper is shown below in Figure 2.

The blocks were moved to the rotary cutter by the action of the chopper feeding mechanism's rod, with a preset feed rate along the guide planes. The guiding planes of the IBF-1 working chamber were installed and fixed in accordance with the dimensions of the experimental blocks of frozen meat. The chopped meat was extracted from the collection tank and subjected to microstructural studies carried out in accordance with **GOST 31479 (2012)** 'Meat and meat products.

Method of histological identification of the composition'. Histological preparations were made on a MIKROM HM 525 freezing microtome. The plane sections were studied using an Axio Imager.A1 light microscope (Carl Zeiss, Germany) using computer software for image analysis. The active power consumed by the electric drive of the installation's cutting mechanism in the operation mode was measured and recorded by an AFM-3192 industrial analyzer-recorder.

Statistical analysis

STATISTICA 10.0 software was used in this study for the statistical analyses. The results were calculated as "middle-value ±standard error" (M ±SE). The statistical distribution was equalized with the application of the theoretical (normal) distribution. The equalization was performed by Pearson's criterion **(Book, Velleman and Veaux, 2007; Greenwood and Nikulin, 1996)**. Differences with p-values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Figure 3 (a, b, c) shows the microstructure of minced meat chopped by obtained by the chopper IBF-1 with the rotary cutters in which technical characteristics are given above.

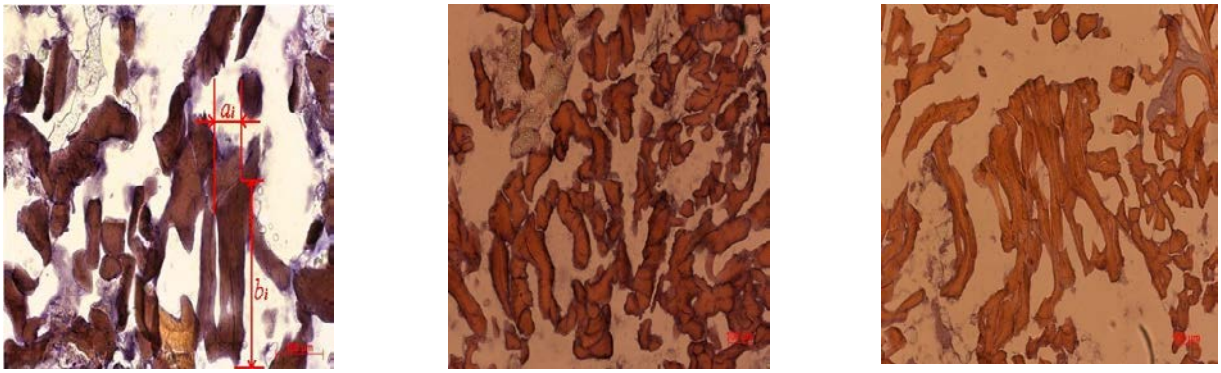
The meat chips size obtained from a certain type of rotary cutter and the applied cutting mode of raw materials were statistically analyzed in the following sequence:

1. The data of microstructural analysis of minced meat was grouped into statistical series (Table 1); **(Gaidyshev, 2001; Lagutin, 2007; Protasov, 2005)**.

If the thickness of the meat chips hits between two adjacent ranges, then the number of hits is divided equally between these ranges **(Gaidyshev, 2001)**. The frequency of hits in the range is defined as the quotient between the number of hits in this range and the total number of meat chip thickness measurements. The average value of the meat chips thickness in the range is determined as the arithmetic mean of the measured data for this range.

2. Using the data given in Table 1, the numerical distribution of the meat chips thickness was presented as a histogram according to the selected ranges of the values (Figure 4).

3. By dividing of every frequency of hit into the *i*-th range p_i^* on the stretch of every appropriate range $\Delta_i = a_{i+1} - a_i$, where a_{i+1} and a_i are the selected limits of the range, we get a table of frequency densities f_i^* (refer to the table 2), by which the histogram of the frequency density distribution f_i^* was built according to the range of values of the meat chips thickness (Figure 5); **(Gaidyshev, 2001; Lagutin, 2007; Protasov, 2005)**.



a b c

Figure 3 Microstructure of the minced meat. Note: a – chopping of beef with a fine teeth cutter, refer to the thickness of meat chips, width of meat chips; b – chopping of pork with a large teeth cutter; c – chopping of beef with hard metal rotary cutter.

Table 1 Grouped statistical series of the meat chips thickness measurements.

Range and its limits, um	The value of the meat chip thickness within the range	Frequency of getting within the range p_i^*	Average thickness of meat chips within the range a_{isr}, um
Range 1; 14 – 20	13	0.062	16.92
Range 2; 20 – 26	16.5	0.078	23.58
Range 3; 26 – 30	18.5	0.088	29.56
Range 4; 30 – 35	22	0.104	32.31
Range 5; 35 – 40	28	0.133	37.26
Range 6; 40 – 45	25	0.119	42.34
Range 7; 45 – 50	26	0.123	47.43
Range 8; 50 – 55	15	0.071	52.27
Range 9; 55 – 60	18	0.085	57.40
Range 10; 60 – 66	12	0.057	62.61
Range 11; 66 – 76	8	0.038	70.77
Range 12; 76 – 91	7	0.033	83.50

The presented statistical analysis refers to beef chopping with a fine-tooth rotary cutter at a rotation frequency of 2,289.14 min^{-1} and a rate of meat block feed per cutter = 0.0243 $m.s^{-1}$. The experimental data were used to calculate the estimates of the sample mean values of the thickness and width of meat chips $a^*_{average}$ and $b^*_{average}$, as well as estimates of the standard deviations of the thickness and width of meat chips σ_a^* and σ_b^*.

In the considered case the values of these parameters were as follows: $a^*_{average}$ = 42.28 um and $b^*_{average}$=166.64 um, σ_a^* = 15.86 um, σ_b^* = 90.48 um. In the same way we calculated the same parameters of the experimental distributions obtained when the rotary chopper IBF-1 was equipped with the other types of cutters and the certain cutting mode for raw materials was used. In particular, when equipping the chopper with a large-teeth cutter, the parameter values were: $a^*_{average}$ = 118.99 um and $b^*_{average}$=495.28 um, σ_a^* = 61.15 um, σ_b^* = 245.57 um; the rotary cutter speed = 2,289.14 min^{-1}; rate of the meat (pork) block feed per cutter = 0.0243 $m.s^{-1}$. When equipping the chopper with hard metal plates cutter, the values of the parameters were: speed of the cutter rotation = 2.033.50 min^{-1}; rate of the meat (beef) block feed per cutter = 0.0243 $m.s^{-1}$.

Calculations show that the experimentally obtained statistical distribution of the meat chips thickness is equalized with the help of the theoretical (normal) distribution. The equalization was performed according to Pearson's criterion **(Kobzar, 2006)**. It shall be noted that the mathematical expectation of the distribution of the specified values very well correlates with the supply of raw materials to the cutter tooth. At the same time, the statistical distribution of the meat chips width does not comply with the Gaussian law. This is explained by the prevailing influence of the cutting edge track on the chips width distribution when the rotary cutting edges of the cutter teeth slide on the surface of the meat block **(Book, Velleman and Veaux, 2007; Greenwood and Nikulin, 1996)**. The size of the track varies widely every moment of the chopping process for every tooth of the rotary cutter, while simultaneously taking part in the raw material chopping. This fact is proved by sweeping the lateral surface of a cylindrical rotary cutter with a helical tooth on the cutting surface of the meat block. Thus, to reduce the dispersion of the resulting width of the meat chips, it is necessary to use the rotary cutters with fragmentation of the rotary cutting edges into the equal sized segments (so called "corn-shaped" rotary cutters), which will equalize the width of the meat slices being cut off with all teeth of the rotary simultaneously during the operation.

The moment of resistance to chopping, i.e. the load on the cutter in the operating mode, varies significantly in magnitude. This is caused by the significant heterogeneity

of the initial raw material as noted above. When changing the load on the milling cutter in the operating mode, the frequency of its rotation changes, which leads to additional dispersion of the linear dimensions of the meat chips due to changes in the parameters of the cutting mode (its feed into the rotary cutter tooth and cutting speed of the raw materials). This factor of influence on the degree of meat chopping reduces the homogeneity of the minced meat produced, which leads to a decrease in the quality of the meat products **(Thalhammer, 1998)**.

Given this, it is necessary to ensure the stabilization of the parameters of the meat-cutting mode by means of an ACS for the chopping process in the operating mode of the cutter. The use of automatic control of a single-stage technological process of minced meat production is shown in the example of a line developed for the production of sausages (Figure 6).

When implementing this project, we set the task not only to improve the technological process of cutting frozen meat raw materials through the use of a new method of chopping by the milling method, but also to ensure the production of minced meat of a preset quality on an automatic line.

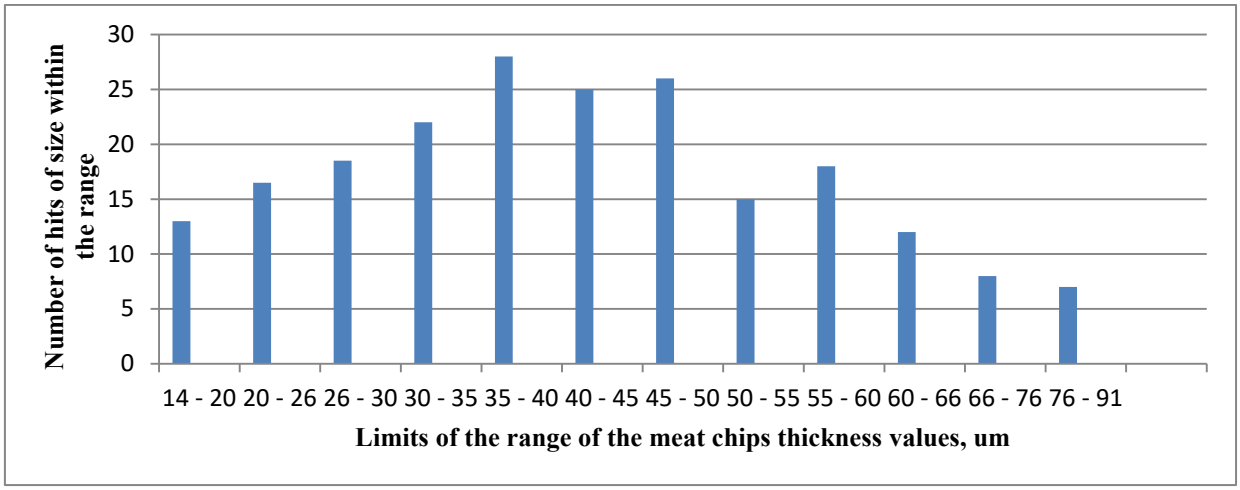


Figure 4 Histogram of the numerical distribution of hits of the meat chips thickness in the selected range of its values.

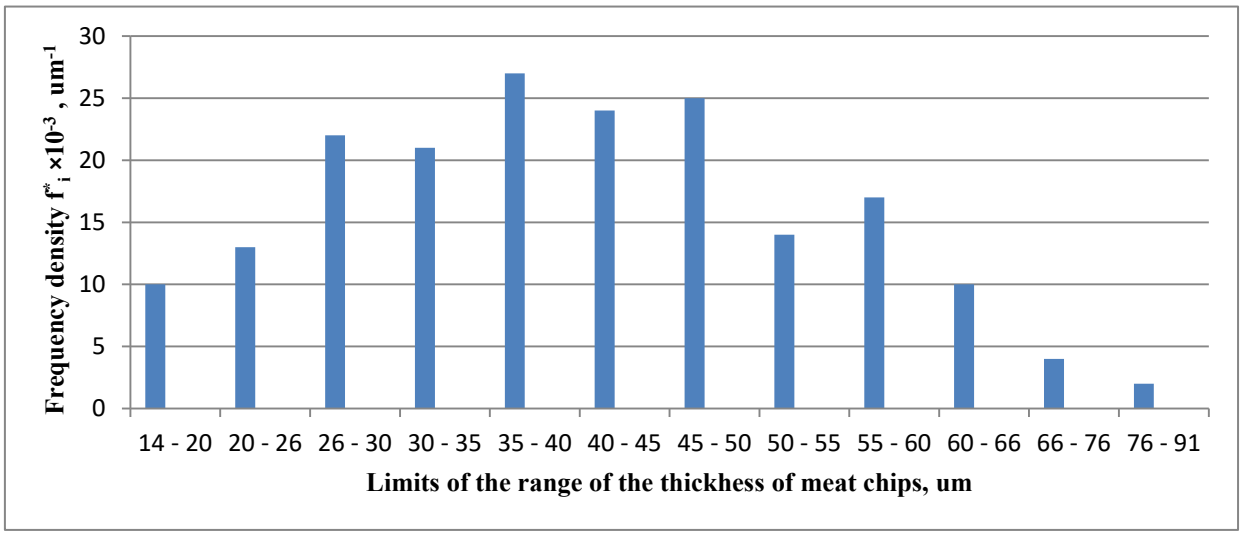


Figure 5 Histogram of the frequency density distribution fi * of the meat chips thickness hitting into the range of its values.

Table 2 Frequency densities f_i^* by range of values of the meat chips thickness.

Range a_i , um	14 – 20	20 – 26	26 – 30	30 – 35
Frequency densities $f_i^* \times 10^{-3}$, um^{-1}	10	13	22	21
Range a_i, um	35 – 40	40 – 45	45 – 50	50 – 55
Frequency densities $f_i^* \times 10^{-3}$, um^{-1}	27	24	25	14
Range a_i , um	55 – 60	60 – 66	66 – 76	76 – 91
Frequency densities $f_i^* \times 10^{-3}$, um^{-1}	17	10	4	2

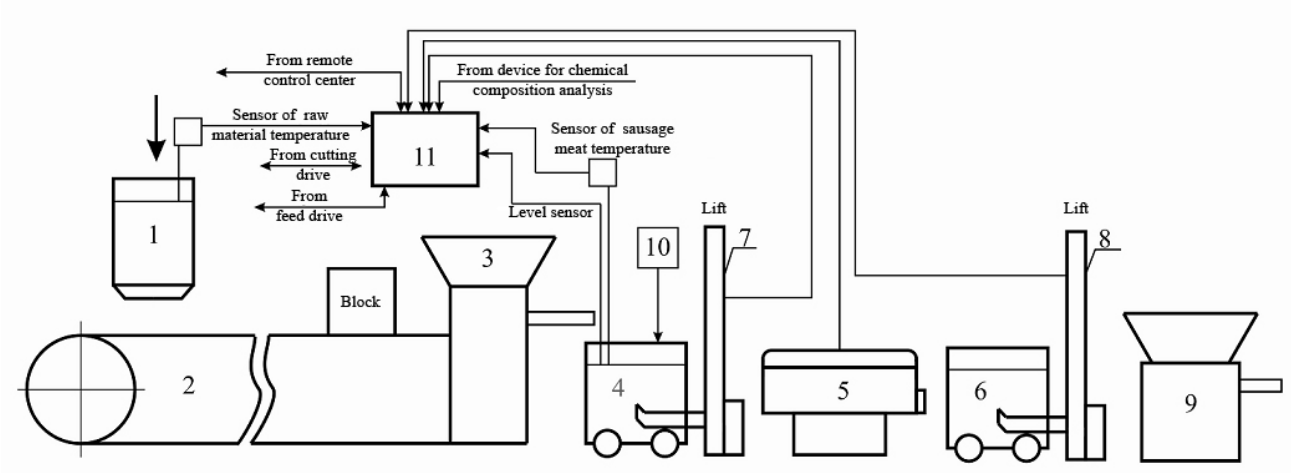


Figure 6 Automatic line for sausage production. 1 – instrument for input control of raw materials; 2 – conveyor belt; 3 – rotary cutter for meat chopping; 4 – storage and dispenser tank; 5 – minced meat mixer; 6 – trolley; 7, 8 – elevators; 9 – filling machine (syringe); 10 – device for analysis of the chemical composition of the chopped meat; 11 – control system computer (CSC).

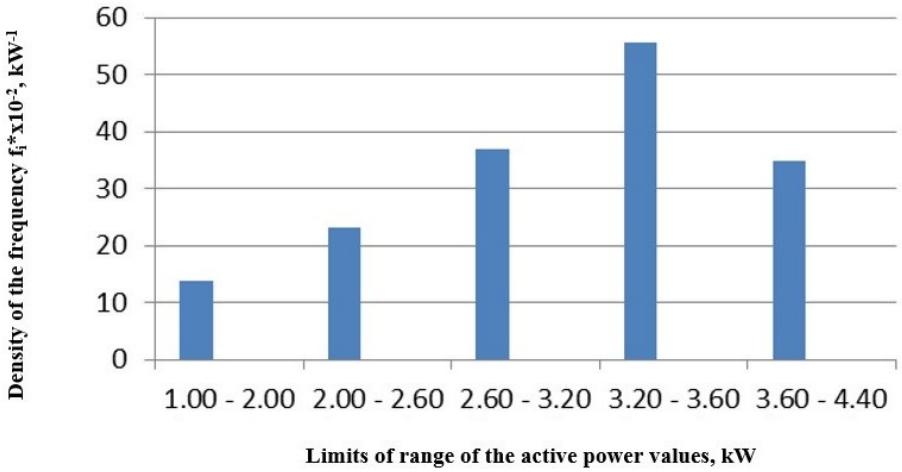


Figure 7 Histogram of the frequency density of the power distribution consumed by the electric motor of the rotary cutting mechanism drive in IBF-1 installation, operating mode, according to the range of its measured values.

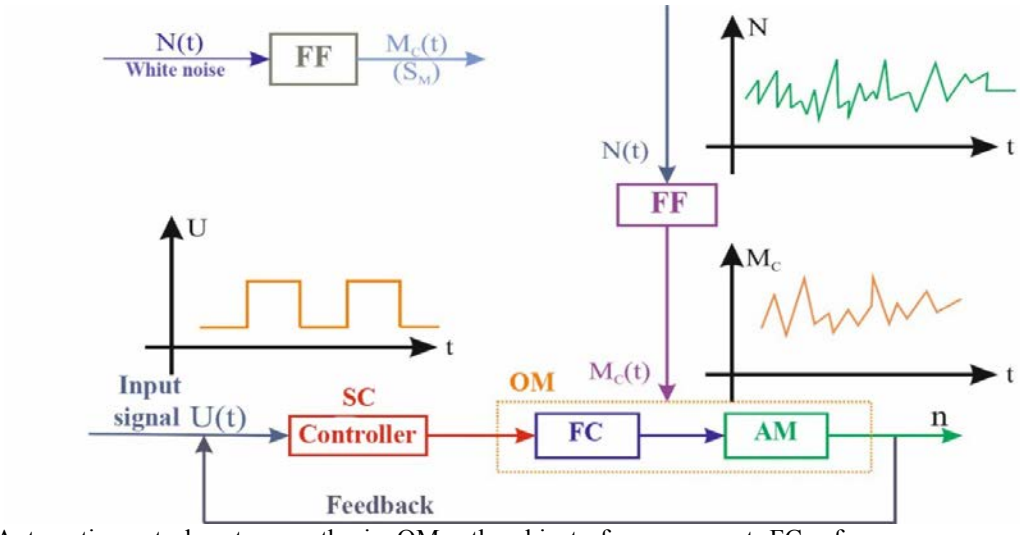


Figure 8 Automatic control system synthesis. OM – the object of management; FC – frequency converter, changing the rotation of the rotary cutter; AM – asynchronous electric motor of the drive on the cutting unit of the chopper; N(t) – white noise; U(t) – input signal (cutting speed setting of the raw material); FF – forming filter; SC – speed controller (frequency) of rotary cutter rotation; Σ – summarizer; n – frequency of the rotary cutter rotation.

To solve this problem, the line is equipped with a storage and dispenser tank, in which the required amount of chopped raw materials of each type (beef, pork, fat) is measured in accordance with the recipe of the finished product. The line also has equipment for monitoring the chemical composition of chopped meat (protein, fat and moisture content) in the production flow in real-time mode. In the process of mixing minced meat in the mixer, its viscosity is controlled by the appropriate sensor and the pH of the minced meat is also measured; water and other components provided by the recipe are dosed and added. The measurement data are sent to the control system computer (CSC – industrial computer), which controls the technological process of producing sausages on the automatic line.

The presented statistical analysis (Figure 7) refers to a beef chopping with a fine teeth rotary cutter at rate of 2.289.14 min^{-1} and a feed rate of a meat block per cutter = 0.0243 $m.s^{-1}$. When constructing the ACS as technological process with the function of stabilization of the operating parameters of one-stage chopping, we can assume that the random process of loading on the cutter is a stationary process with the ergodicity property. This means that any realization of a stationary random process of certain duration represents to a sufficient degree the entire set (ensemble) of realizations of the process considered.

Taking into consideration the normal law of load distribution on the rotary cutter during the chopping process, for mathematical modelling of this disturbance (M_c in Figure 8) it is expedient to use the forming filter (FF) method **(Pupkov et al., 2004c)**.

The FF method is based on the fundamental property of a random stationary process passing through a linear system, which can be expressed as follows **(Gaidyshev, 2001; Kobzar, 2006)**:

$$S_{y(\omega)} = \left|W_{ff(j\omega)}\right|^2 \cdot S_{x(\omega)} \quad (3)$$

where $S_y(\omega)$ is the spectral density of the random process at the system output; $S_x(\omega)$ is the spectral density of the random process at the system input; $|W_{ff}(j\omega)|^2$ is the square of the system transfer function module; and ω is the frequency.

We will consider white noise as a random process at the input. White noise in the theory of stochastic processes (TSP) is understood as the limiting case of a sequence of very short pulses, the amplitude of which is an independent random variable value with a very large dispersion, and the ratio of the dispersion of these pulses to the frequency of their appearance is a constant (finite) value **(Gaidyshev, 2001; Ivashov et al., 2018)**. From the definition of white noise, it follows that the spectral density of the input random process is constant through the entire frequency range and is equal to:

$$S_x(\omega) = S_0 = const$$

If we assume S_0 = 1, then formula (3) will have the following form:

For synthesis of the ACS technological process on the proposed line (in particular solving the task of predicting the size of meat chips in the chopping process), it is necessary to take into account the impact on the rotary cutter from the load side. To do this, it is necessary to develop a mathematical model of the load on the rotary cutter in the process of chopping raw materials. The data obtained during the experiment and processed by methods of mathematical statistics give reason to assume, in the first approximation, the normal law of load distribution on the rotary cutter when chopping industrial-size blocks of raw materials. The experimental statistical distribution of consumption of active power of the cutting mechanism IBF-1 in the chopper drive (Figure 7) is equalized with the help of the theoretical distribution according to the Gaussian law.

$$S_y(\omega) = \left|W_{ff}(j\omega)\right|^2 \cdot S_x(\omega) = \left|W_{ff}(j\omega)\right|^2 \cdot 1 = \left|W_{ff}(j\omega)\right|^2 \quad (4)$$

Thus, in order to realize a random process with a given statistical characteristic, i.e. a given spectral density $S_y(\omega)$, we must send the white noise with a unit spectral density through a linear system with a transfer function $W_{ff}(j\omega)$. Such a linear system in the theory of automatic control is called a shaping filter.

Single-stage chopping control provides automatic maintenance of the set degree of chopping of raw materials while stabilizing the parameters of the cutting mode in real time. The ACS of the technological process on the developed automatic sausage production line has the features of an intelligent control system **(Pupkov et al., 2004a)**: 1) close interaction of the intelligent system's information with the environment when using information communication channels – in the case of control over the single-stage chopping of meat blocks, their temperature, the presence of foreign particles, and the weight of the units are controlled; 2) the availability of forecasts of changes in the external environment and their own behaviour – in this case, the system calculates the forecast amount of meat chips obtained by cutting of the raw materials with a multiblade tool; 3) improving intelligence and improving one's own behaviour – the system is trained in real time, updating the forecast amount of meat chips in the process.

The ACS algorithm (Figure 9) is characterized by the fact that, in addition to the statistical information obtained during chopping of a real block of meat about the change in cutting shaft speed under the influence of the chopping resistance moment, the CSC will have an additional volume of similar statistical information while computer modelling of chopping of 'virtual' blocks of meat.

This allows the CSC to calculate the point and interval estimates of the process under changing rotation speed of the chopper cutting shaft in the chopping process, and on their basis, according to the established analytical dependence, it can determine the same estimates for changing the characteristic size of the resulting meat chips with a preset statistical accuracy and reliability.

The CSC also calculates the variance of these estimates themselves, i.e. determines the degree of 'blurring' of the range limits which include the size of meat chips, which is important in the production of baby food.

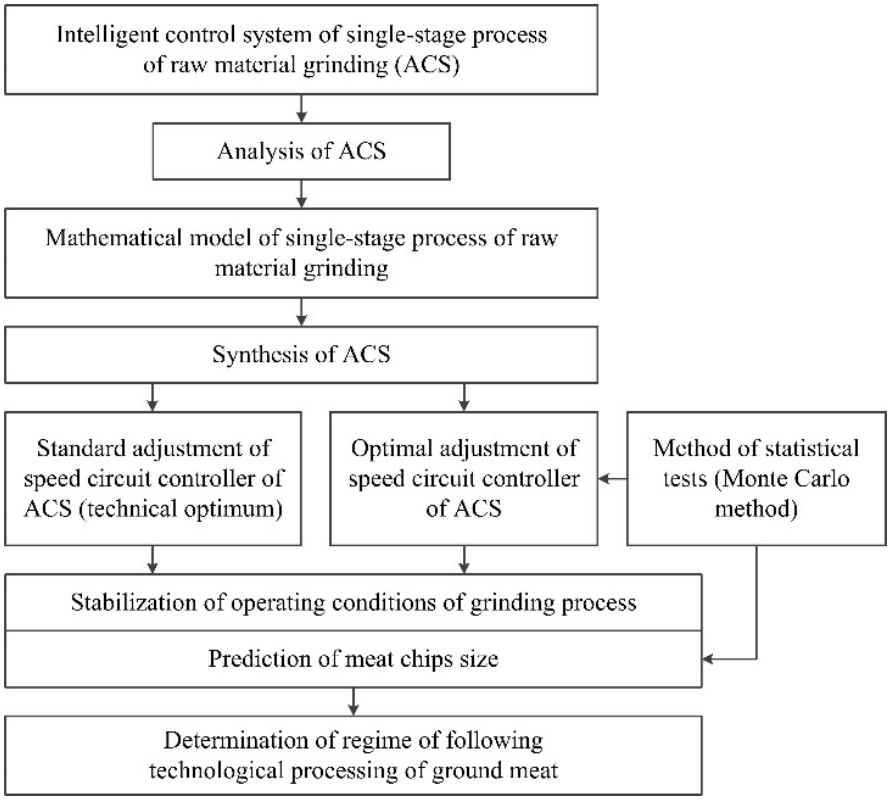


Figure 9 Block diagram of the development of an automatic control system (ACS) for a single-stage process of chopping of frozen meat raw materials.

In the process of real chopping of a block meat, the CSC accumulates and processes statistical information about the chopping process, i.e. the system is trained in the operating mode, improving its prediction of the degree of the raw material chopping. As a result of implementing the above algorithm, the control system will have statistical information about the degree of raw material chopping in an explicit (digital) form. The composition of the ACS may include equipment for rapid analysis of the chemical composition of chopped meat, as well as its temperature after chopping. Then, the further process of minced meat production can be controlled strictly as a function of time, excluding the subjective factor of assessing the degree of the final product readiness. This will allow acquisition of a finished product with a preset high quality.

CONCLUSION

In the proposed project for automatic sausage production lines, the technological chain is reduced in comparison with the traditional solution: instead of three meat-cutting machines: block cutting machine → the top → the cutter, only one is used – the rotary cutter for meat chopping. This significantly reduces the cost of operating meat processing plants engaged in the production of mass market products. Machine control of the minced meat produced for sausages at all stages of its production ensures high quality of the final product. Full automation of the technological process of minced meat production using the proposed control system opens the way to the design of automatic meat processing plants in the future.

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THE STUDY OF SELECTED COMPONENTS OF GRAPE AND FRUIT WINES

Marcela Cehula, Mojmir Baron, Tunde Jurikova, Adrian Alumbro, Methusela Perrocha, Ivo Ondrasek, Jiri Mlcek, Anna Adamkova, Jiri Sochor

ABSTRACT

This study aimed to compare chemical properties, in terms of the content of volatile substances, antioxidant compounds, and antioxidant activity (AOA), in samples of fruit and grape wines. For this purpose, the following types of wine were selected, namely fruit wines (apple, strawberry, and elderberry) and grape wines (Müller-Thurgau, St. Lawrence Rosé, and Blue Portugal). Basic analyses of fruit and grape wines were conducted by using the ALPHA method and volatile substances in wines were determined by the GS/MC method. The antioxidant content and AOA were estimated by spectrophotometry, using two types of DPPH method. The results of the experiment showed that the highest values of antioxidant compounds (anthocyanins and flavanols) were found in the samples of Blue Portugal wine and elderberry wine. Significant differences were determined among the wines in antioxidant content, which may have been influenced by the production technology. The results showed that the alcohol content of the wines ranged from 10.99% to 19.49% vol. The highest alcohol content was measured in the elderberry wine samples and the lowest in those of the apple wine. The strawberry wine had the highest titratable acid content, which corresponded to a pH of 3.38. The lowest content was measured in the apple wine samples. Due to the higher acid content of the strawberry wine, a higher residual sugar level of 46.9 $g.L^{-1}$ was obtained. We noted that the red fruit wines contained a higher proportion of valuable bioactive substances than white grape wines. Wines with superior sensory properties did not contain higher levels of antioxidants or higher AOA. The research results can provide a helpful reference for the widespread use of grape and fruit wines in medical, nutritional, and other fields.

Keywords: antioxidant; analytical parameter; fruit wine; grape wine; volatile substance

INTRODUCTION

Wines produced in the Czech Republic have been among the most highly valued in terms of worldwide parameters, as evidenced by several international awards received by winemakers. The success of winemakers is also valued very positively by consumers, who are starting to prefer wines produced in the Czech Republic over foreign wines **(Snopek et al., 2018)**.

The main target of producing quality and premium wines is expressing the connection, through sensory character, to the place where the grapes were cultivated **(Slaghenaufi et al., 2019)**. Similar to other foods, wine has a taste and aroma that significantly affect the interest of the final consumer. These characteristics of the wine are influenced not only by the cultivar used but also by how the wine has been stored during its maturation. The most valuable compounds in wine are volatile organic and bioactive compounds.

The most studied compounds from this group are terpenes **(González-Barreiro et al., 2015)**. Different methods of wine-making technology have affected substance levels **(Baron et al., 2018)**. Polyphenols, especially resveratrol, anthocyanin, and catechins, are the most valued wine antioxidants **(Snopek et al., 2018)**.

The current modern trend has been in plant breeding. One scientific area that has been discussed has concentrated on producing large-fruited cultivars and clones with regular fertility and a higher profile of bioactive substances and AOA. After several years of exploring this issue, crop refinement has only been achieved by selecting the most usable and fastest-growing plants. Eventually, the selection of plants studied has influenced the morphological and quantitative properties of crops all around the world **(Kaczmarska et al., 2015; Cehula et al., 2019)**.

Various polyphenolic compounds are valued for plant cell pigmentation, i.e. anthocyanidins, responsible for red, blue, violet, orange, and purple colors **(Moreno-Montoro et al., 2015; Vallverdu-Queralt et al., 2015; Barba et al., 2016)**.

The aim of this study was the comparison of the different bioactive substance content of grape wines and fruit wines. Three kinds of grape wine (‘Müller-Thurgau’, ‘ St. Lawrence Rosé’ and ‘Blue Portugal’) and three kinds of fruit wine (strawberry, elderberry, and apple wine) were selected. During the study, attention was given to the

evaluation of AOA by the DPPH method, estimation of the total content of anthocyanins, flavanols, acetic, citric, lactic, malic and tartaric acids, alcohol, fructose, glucose, sacharose, and glycerol, of pH, of total acidity, and sugar content.

Scientific hypothesis

H1: The content of antioxidants depends on the type of wine.

H2: Red wines contain higher levels of antioxidant compounds than white wines.

H3: Wines with significant sensory properties contain more antioxidants than other wines.

H4: By monitoring the occurrence of volatile substances, we can show that grape wines will have a higher content than fruit wines.

MATERIAL AND METHODOLOGY

Characteristics of selected wines:

Müller-Thurgau, 2014: Samples were obtained from the Kubík winery (Velké Bílovice, Czech Republic). It is a high quality, fresh, young wine with a typical yellow-green color, spicy taste, and somewhat pleasant smell.

St. Lawrence Rosé, 2014: The samples were obtained from the same winery. It is a quality deep pink wine, with tones of strawberries in aroma and taste.

Blue Portugal, 2014: We obtained the samples of this wine from the Veverka winery (Čejkovice, Czech Republic). The wine is a soft ruby color, with the aroma of flowers. The tannin content provides it with an attractive expression.

Strawberry wine: This is made from the strawberry *Fragaria annanassa*, cultivar ´Karmen´. The fruits were stripped and crushed and were allowed to macerate for 12 hours before the mash was pressed. The juice was drained by sedimentation. Water and sucrose were added to achieve the desired acid reduction. Nitrogenous substances were also treated with nutrient salt. The juice was then inoculated with noble yeast and placed in an appropriate glass container, fitted with a fermentation plug, and allowed to ferment. After fermentation, the wine was removed from the sludge and filtered. Subsequently, the wine was bottled.

Elderberry wine: The wine was made from elderberries (*Sambucus nigra*, L.). The elderberry fruits were stripped and crushed. The mash was heated to 80 °C for 15 minutes, to destroy the sambunigrin, which causes headaches and nausea. Then the mash was macerated for 48 hours and subsequently pressed. The juice was drained by sedimentation. Water and sucrose were added to achieve the desired acid reduction. Nitrogenous substances were also treated with nutrient salt. The juice was then inoculated with noble yeast and placed in an appropriate glass container, fitted with a fermentation plug, and allowed to ferment. After fermentation, the wine was removed from the sludge and filtered, then subsequently bottled.

Apple wine: The wine was made from the cultivar ´Jonagold´. The apples were stripped of stalks and cores, then crushed. The mash was pressed, and the juice was drained by sedimentation. Water and sucrose were added to achieve the desired acid reduction. Nitrogenous substances were also treated with nutrient salt. The juice was then inoculated with noble yeast and placed in an appropriate glass container, fitted with a fermentation plug, and allowed to ferment. After fermentation, the wines were removed from the sludge and filtered, then, subsequently, was bottled.

For our research we used the followed methods:

Determination of antioxidant activity by the DPPH method

For the determination procedure, see the description earlier **(Sochor et al., 2010)**. We mixed 150 µL volume of the reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl; $DPPH^{\bullet}$) with 15 µL of wine sample. Each process was repeated twice. All samples were incubated for 10 minutes. After incubation, we measured samples at an absorbance of 505 nm on the spectrometer. The results were expressed as trolox equivalents.

Estimation of anthocyanin content

These measurements were performed using recognized spectrophotometric methods **(Zoecklein et al., 1999)**. The wine sample was placed into a 0.2 cm path-length quartz cuvette, then 200 µL of the sample and 1.8 mL of 1.1 M HCL were added and the resulting solution was thoroughly mixed and kept at room temperature for 180 minutes. A 0.22 M solution of $K_2S_2O_5$ was used as a blank.

The absorbance was read at 520 nm (A_{520}^{HCl}) for anthocyanins. Total concentrations of anthocyanin ($mg.L^{-1}$) were calculated as follows:

$$\text{Total content of anthocyanins } (mg.L^{-1}) = 4 \times \text{dilution} \times [A_{520}^{HCl} - (5/3) \times A_{520}^{SO2}]$$

Estimation of total flavanol content

Total flavanol content was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method **(Li, Tanner and Larkin, 1996; Tomášková et al., 2017)**. Compared with the widely used vanillin method, a major advantage of this method is that there is no interference from anthocyanins. Furthermore, it provides higher sensitivity and greater specificity. Wine (20 µL) was poured into a 1.5 mL Eppendorf tube and 980 µL of DMACA solution (0.1 % in 1 M HCl in MeOH) was added. The mixture was vortexed and allowed to react at room temperature for 12 minutes. Absorbance at 640 nm was then read against a blank sample prepared similarly but without DMACA. The total flavanol concentration was then estimated from a calibration curve and constructed by plotting known solutions of catechin (1 − 16 $mg.L^{-1}$) against A_{640} ($r^2 = 0.998$). The results were expressed as $mg.L^{-1}$ of catechin equivalents.

GC-MS determination of individual volatile compounds and sample preparation

The concentration of individual volatile substances in the wine was determined by the previously unpublished method of extraction with methyl t-butyl ether. The following was pipetted into a 25 mL volumetric flask: 20 mL of wine, 50 µL of 2-nonanol solution (500 $mg.L^{-1}$), and cyclopentanone (25 $g.L^{-1}$) in ethanol as the internal standard, and 5 mL of saturated $(NH_4)_2SO_4$ solution.

Table 1 Determination of basic analytical parameters in selected wines.

Type of wine/ parameter	Alcohol (%)	Titr. acids ($g.L^{-1}$)	Red. sugar ($g.L^{-1}$)	pH	Malic acid ($g.L^{-1}$)	Lactic acid ($g.L^{-1}$)	Acetic acid ($g.L^{-1}$)
Müller-Thurgau	12.31	7.07	7.6	3.49	3.76	0.16	0.26
St. Lawrence rosé	13.22	7.73	7.1	3.4	3.71	0.39	0.34
Blue Portugal	13.41	5.14	0.1	3.54	0.02	2.15	0.51
Apple wine	10.99	3.47	8	3.25	3.22	0	0
Strawberry wine	16.7	8.47	46.9	3.38	3.13	1.12	0.34
Elderberry wine	19.49	4.75	15.2	3.98	3.43	0.5	0.39
SD	±6.14	±2.91	±16.19	±1.35	±1.69	±0.78	±0.19
	Tartaric acid ($g.L^{-1}$)	**Glycerol ($g.L^{-1}$)**	**Glucose ($g.L^{-1}$)**	**Fructose ($g.L^{-1}$)**	**Sucrose ($g.L^{-1}$)**	**Density**	**Citric acid ($g.L^{-1}$)**
Müller-Thurgau	2.83	7.18	3.1	5.51	0.07	0.99	0
St. Lawrence Rosé	2.61	6.4	1.94	5.72	0	0.99	0.03
Blue Portugal	2.47	9.64	0.61	0.24	0.02	0.99	0.05
Apple wine	0.98	7.07	1.65	7.65	0	0.99	0.44
Strawberry wine	0.49	11.93	2.99	44.97	0	1	2.67
Elderberry wine	0	12.22	1.11	13.97	0	0.99	0.08
SD	±1.26	±4.16	±1.16	±15.64	±0.03	±0.38	±0.98

Note: Experiment was replicated 3 times.

The flask contents were then thoroughly mixed, and 0.75 mL of extraction solvent added, MTBE with the addition of 1% neohexane. After thorough mixing of samples, and separation phases, the upper organic layer was transferred to a microtube with a portion of the resulting emulsion, centrifuged and the clear organic phase was dried over anhydrous magnesium sulphate. We continued using the treated extract for GC-MS analysis.

Estimation of acetic, citric, lactic, malic and tartaric acids, alcohol, fructose, glucose, sacharose, and glycerol, of pH, of total acidity, and the sugar content

The aforementioned parameters were estimated using the ALPHA apparatus (Bruker, Germany). The ALPHA spectrometer is a compact FTIR analyser based on the principle of ATR sampling. Physical-chemical parameters of wine samples were determined according to the **EEC Official Method by the European Commission (2003)**, which was used in a similar study by **Condurso et al. (2018)**. This method of sampling considerably simplifies the preparation of samples for analysis. This means that samples of clarified wine were analysed directly, (i.e. without any adjustments), while those of musts and fermenting wine were centrifuged at 13 400 rpm for six minutes. Before the measuring of the first sample, the apparatus was thoroughly rinsed with distilled water and the background was measured using deionized water as a blank sample. For analysis, 1 mL of clear wine was sampled with a syringe; of this, one half (0.5 mL) was used for the rinsing of the system, and the remaining half (i.e. also 0.5 mL) was used for the subsequent three measurements. Depending on the method of calibration (musts/fermenting wine/fermented wine), the recorded resulting amounts were automatically evaluated by software.

Separation conditions

Column: DB-WAX 30 m x 0.25 mm; 0.25 μM stationary phase (polyethylene glycol). Sample injection volume: 1 μL split ratio 1:5; carrier gas flow rate He:1 ml/min (linear gas velocity 36 $cm.S^{-1}$); injection chamber temperature: 180 °C. The initial column chamber temperature of 45 °C was maintained for 3.5 minutes, followed by a temperature gradient: up to 90 °C by 12 $°C.min^{-1}$ held for 0.75 minutes, up to 120 °C by 3 $°C.min^{-1}$.

Statistic analysis

For this study, we applied ANOVA, simple descriptive statistical methods, and correlation- regression. Statistical analysis was performed using NCSS 2019 software (ver. 19.0.4.; NCSS, LLC Utah, USA).

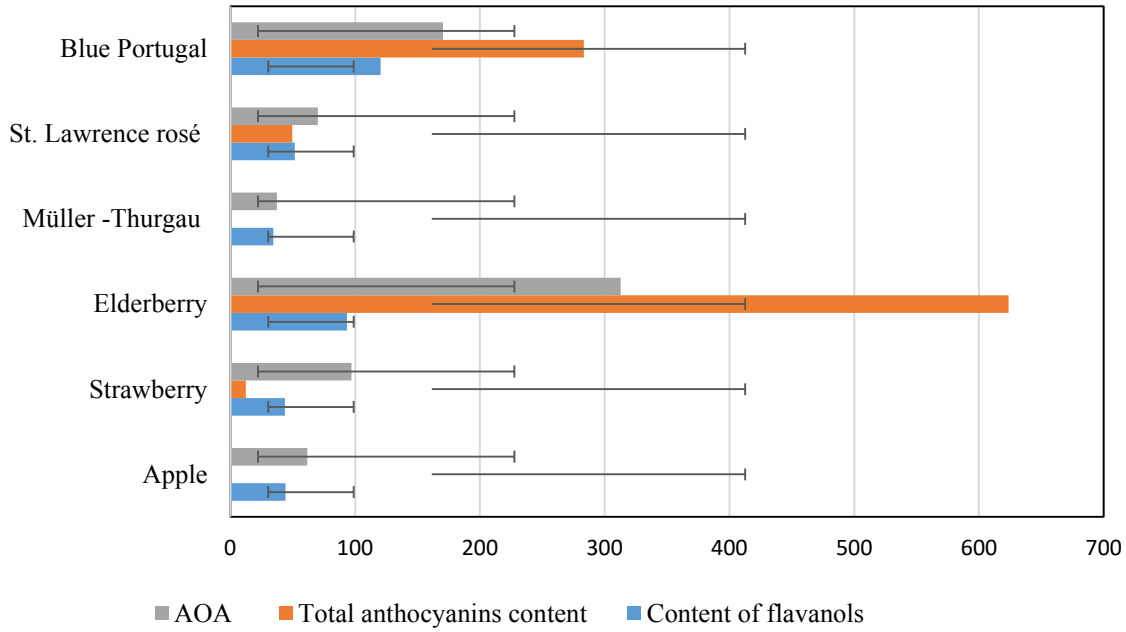

Figure 1 Estimation of Antioxidant activity – AOA (equivalent to TROLOX mg.L^{1}); total anthocyanins content (mg.L^{-1}) and content of flavanols (mg.L^{-1}). Note: Experiment was replicated 3 times.

RESULTS AND DISCUSSION

Determination of basic analytical parameters in selected wines

The results are shown in Table 1. The highest percentage of alcohol was determined in the elderberry wine samples, while the smallest percentage of alcohol was measured in the apple wine samples. The pH range was from 3.25 (apple wine) to 3.98 (elderberry wine). The lowest content of malic acid was determined in the samples of Blue Portugal and the highest content identified in the samples of Müller-Thurgau.

Lactic acid was maximal level in the samples of Blue Portugal, and minimal level in the apple samples. Zero acetic acid content was determined in the case of the apple wine and tartaric acid content in the elderberry wine samples.

On the contrary, the highest values were measured in the Blue Portugal (for acetic acid) and the Müller-Thurgau (for tartaric acid).

We also measured the value of zero in citric acid content, namely in the Müller-Thurgau, very low values in the St. Lawrence rosé and Blue Portugal, and high values in the strawberry wine. We found that the lowest values were of sucrose, of which we measured values close to zero, but we were able to determine higher fructose levels. The values of glucose were from 0.61 to 3.10 g.L^{-1}.

We observed that in the case of the grape wines, the analytical parameters were generally higher than in the case of the fruit wines. Among the grape wines, due to the analytical parameters, we evaluated Blue Portugal as the highest and, among fruit wines, we found that these values were the highest in the elderberry wine.

In the study by **Robles et al. (2019)**, some organic acids were determined and identified in samples of wine. The study mentions that the following organic acids are found in Czech white grape wines: tartaric acid (4.4 – 17.4 mM), malic acid (12.4 – 46.4 mM), acetic acid (1.7 – 27.9 mM), and lactic acid (1.4 – 27.4 mM). The values of tartaric acid measured in our study were in the above range. The content of malic acid in the observed samples was below the levels of those in the above study. Only samples of Blue Portugal corresponded in a range of values of lactic acids. There were no similarities in the other grape wine samples to those in comparable research.

Higginson et al. (2015) demonstrate in their study that single berry samples taken from bunches (grape) showed a large variation in the content of tartaric acid and of malic acid across a single vine. This assayed variation is relevant for studies collecting samples of berries for organic acid analysis. We noted that grape berries (but not fruit) predetermined chemical and sensory properties.

Estimation of antioxidant activity, total anthocyanin content and flavanol content

The next aim of this study was to determine AOA, total anthocyanin content, and flavanol content in all the wine samples. After using statistical analysis methods, we came to the following conclusions:

The highest AOA was measured in the elderberry samples and the lowest content in the Müller-Thurgau samples.

Elderberry wine was the highest-scoring sample regarding total anthocyanin content. Otherwise, low levels were measured in the Müller- Thurgau and apple wine samples. The highest values of flavanol content were found in the Blue Portugal and elderberry wine samples, whereas the lowest values were determined in the Müller-Thurgau. All results are shown in Figure 1. Considering the above-measured parameters, we concluded that elderberry wine and Blue Portugal proved to be the wines with the highest content. Conversely, the lowest levels of

measured parameters were demonstrated in the Müller-Thurgau and apple wine samples. By using the ANOVA statistical method, we showed a statistically significant difference between individual types of wine (grape and fruit wines; $p \leq 0.05$). The correlation and regression method showed that all represented pairs of amounts appeared on a single line and that the function had a rotating character. We found that the coefficient was equivalent to +1, which ultimately means a greater degree of interdependence.

By comparing the various constituents of the wines, we assessed that, in terms of AOA, fruit wines contained higher values than grape wines. Furthermore, in terms of the total anthocyanin content, we evaluated fruit wines as higher than grape wines. However, the flavanol content increased with red wines, elderberry wine, and Blue Portugal, as confirmed by other studies **(Vilas Boas et al., 2019; Lingua et al., 2015)**.

Bruno and Sparapano (2007) mentioned that the content of each substance in grapes was influenced by abiotic factors (e.g. by variety, parent soils, geographic situation, locality, and climatic conditions). We agreed with this statement and we added agricultural and horticultural methods and interventions in vineyards during the growing season. The studies by **Wirth et al. (2010)** and **Baron et al. (2017)** assert that the bioactive substance content and AOA in wine depends on wine-making technology, e. g. by the pressing, maceration, fermentation, filtration, and/or bottling techniques used. They also declare that these contributions have an effect on the sensory properties of wine, the storage time, and other indicators that promote human health.

The study **Čakar et al. (2019)** aimed to determine the potential of the strawberry and drupe (apricot, plum, and sweet cherry) fruits for the production of new fruit wines Improved with phenolic compounds. They found general results that showed that the same fruit wine samples presented high redox potentials, marginally lower than strawberry, and plum wine samples.

The antioxidative potential and total phenolic content were tested in elderberry must and wine in the study **Schmitzer et al. (2010)**. The level of total phenolic content in elderberry must and wine was 2004.13 GAE.L^{-1} and antioxidative potential of elderberry wine was equal to red wine. There was a detected correlation between total phenolic content and antioxidative potential of elderberry wine. Moreover, examinations of elderberry fruits in other studies showed that it contains high biological activity components, primarily polyphenols, mostly anthocyanins, flavonols, phenolic acids, and proanthocyanidins, as well as terpenes and lectins **(Sidor and Gramza-Michałowska, 2015)**.

Mlček et al. (2019) concluded that adequate wine consumption leads to cancer prevention and another health disease. Studies dealing with the content of bioactive substances in beverages made from berries showed that in addition to beneficial effects on human health, these substances have also side effects, e. g. it may cause an allergic reaction for unexplained reasons **(Sedláčková et al., 2018; Nunes et al., 2019; Snopek et al., 2019)**.

Determination of the content of volatile substances in selected samples of fruit and grape wines

Table 2 shows the measured values of individual volatile substances. The fruit wines contained more methanol than the grape wines. The highest level of ethyl acetate was measured in the strawberry wine and other values were similar. Among the terpenes, the most significant that we identified were in the samples of strawberry wine (linalool 100 μg.L^{-1}; α-terpineol 43 μg.L^{-1} and acetoin 9.7 mg.L^{-1}); compared to the strawberry wine, elderberry wine contained more acetoin, and apple wine was higher in methionol content. **Lan et al. (2017)** found that changes in taste properties in wine occur at an early stage of fermentation, i.e. 0 – 4 days. The content of aldehydes, ketones, heterocyclic and aromatic compounds were very reduced by fermentation. The same opinion was presented in studies **Peng et al. (1997); Lubbers, Verret and Voilley (2001)** and **Spence, Velasco and Knoeferle (2014)**.

Comparing the values of volatile substances in grape wines, we found that Blue Portugal and Müller-Thurgau were wines with a relatively high content of linalool. Low total terpene content was demonstrated in samples of St. Lawrence rosé. Geraniol, methionol, and acetoin were measured; Müller-Thurgau differed substantially from Blue Portugal with the highest content of ho-trienol and α-terpineol. The only value of nerol was detected in the Blue Portugal sample. We definitively demonstrated fruit wines as wines with much greater values of volatile substances

Table 2 GC-MS (mg.L^{-1}) determined individual volatile compounds.

	Müller-Thurgau	St. Lawrence rosé	Blue Portugal	Apple wine	Strawberry wine	Elderberry wine	SD
Methanol (mg.L^{-1})	63.70	48.80	100.20	32.60	83.90	221.40	±71.00
2,3-butanediol (mg.L^{-1})	269.00	372.90	1251.30	210.80	2798.10	5087.40	±1883.12
Butyric acid (mg.L^{-1})	1.40	2.23	0.64	1.30	3.51	0.74	±1.16
Ethyl acetate (mg.L^{-1})	74.70	73.50	62.80	55.50	191.60	72.40	±57.37
Isoamyl acetate (mg.L^{-1})	4.43	2.49	0.18	3.85	1.11	1.45	±1.72
Linalool (μg.L^{-1})	65.00	10.00	70.00	0.00	100.00	28.00	±39.50
Ho-trienol (μg.L^{-1})	24.00	0.00	8.00	0.00	0.00	0.00	±9.07
α-terpineol (μg.L^{-1})	17.00	0.00	15.00	0.00	43.00	0.00	±16.12
Nerol (μg.L^{-1})	0.00	0.00	3.00	0.00	0.00	0.00	±1.13
Geraniol (μg.L^{-1})	10.00	2.00	12.00	0.00	11.00	8.00	±5.30
Methionol (mg.L^{-1})	1.64	0.57	5.56	10.72	5.32	1.97	±3.78
Acetoin (mg.L^{-1})	0.00	6.40	10.50	1.30	9.70	19.20	±7.05

Note: Experiment was replicated 3 times.

than grape wines. Moreover, we found statistically significant differences between the measured values ($p \leq 0.05$).

Dziadas and Jeleń (2010) measured terpenes in white wines (different cultivars). As an example, we chose Riesling. The results showed that linalool reached a level of 16.6 – 54.4 $\mu g.L^{-1}$. Comparing it with Müller-Thurgau, we noted a higher level of linalool. In contrast, the content of α-terpineol was much lower in our examined samples than those of the results from the comparison study. The nerol content, of which almost none of the examined samples allowed an estimate, had been determined with a decisively higher content in the above research. **Tarko et al. (2008)** declared an opinion that red wines had higher antioxidant potential and total polyphenol content than analyzed white grape and fruit wines. The prevailing volatile compounds of wines were higher alcohols, mainly amyl alcohols and isobutanol. Different conditions of double fermentation were examined in a study **Ubeda et al. (2011)**. There was added SO_2 and pectolytic enzymes which increased the level of methanol and acetaldehyde, especially in strawberry purees. In the study **Kong et al. (2019)** results showed that enzyme treatments improved the contents of volatile substances. The levels of terpenes and higher alcohols increased constantly during alcohol fermentation. **Yang et al. (2020)** and **Bhat (2000)** also mentioned the quality of fruit wines is related to the corrected application of enzymes in the process of winemaking.

Ayestarán et al. (2019) studied the effect of the winemaking process on the volatile composition and aromatic profile of Tempranillo Blanco wines.

The results showed that carbonic macerated wines had a higher level of alcohols and carbonyl compounds. We agreed with this statement.

CONCLUSION

We noted that red fruit wines contained a higher proportion of valuable bioactive substances than white grape wines. Wines with superior sensory properties did not contain more important antioxidants or higher values antioxidant activity. However, we stated that white grape wines contained a higher content of volatile substances, but, especially in the red wine samples (grape and fruit), we measured higher values for the research parameters. Relatively few scientific articles are published on fruit wines. Therefore, it was very interesting to evaluate these wines in terms of basic analytical parameters and make a comparison with grape wines.

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DETERMINATION OF VOLATILE ORGANIC COMPOUNDS IN SLOVAK BRYNDZA CHEESE BY THE ELECTRONIC NOSE AND THE HEADSPACE SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Jana Štefániková, Július Árvay, Michal Miškeje, Miroslava Kačániová

ABSTRACT

The aim of the present study was to describe volatile organic compounds of the traditional Slovak bryndza cheese determined by using an electronic nose (e-nose) and a gas chromatography mass spectrometry (GCMS) with head-space solid phase microextraction (HS-SPME). For the first time, e-nose based on the gas chromatography principle with a flame ionization detector was described to identify and quantify aroma active compounds of bryndza cheese from Slovakia. The e-nose detects aroma compounds of very small concentrations in real-time of a few minutes and identifies them by comparing *Kovats´* retention indices with the NIST library. Bryndza cheese produced from unpasteurized ewe´s milk and from a mixture of raw ewe´s and pasteurized cow´s types of milk were collected from 2 different Slovak farms beginning in May through to September 2019. The flavour and aroma of bryndza cheese are apparently composed of compounds contained in milk and the products of fermentation of the substrate by bacteria and fungi. Regarding volatile organic compounds, 25 compounds were detected and identified by an electronic nose with a discriminant >0.900 with ethyl acetate, isopentyl acetate, 2-butanone, acetic acid, butanoic acid, and butane-2,3-dione confirmed by gas chromatography. We confirm the suitability of the electronic nose to be used for monitoring of bryndza cheese quality.

Keywords: Aroma active compounds; Ewe´s cheese; Slovakia; Electronic nose

INTRODUCTION

One of the traditional ewe´s milk production is bryndza cheese or Oštiepok cheese **(Zajác et al., 2019; Šnirc et al., 2019)**. Slovak bryndza cheese is natural, white, spreadable cheese, manufactured according to the traditional method. It is recognized in the European Union by Protected Geographic Indication (PGI) status as cheese produced in specified mountainous regions of Slovakia **(Commission Regulation (EC) No 676/2008; Šaková et al., 2015; Zeleňáková et al., 2016)** where unpasteurized ewe´s milk is processed to obtain the Slovak bryndza cheese. The mountainous regions of Slovakia differ in altitude, climate, geological, and vegetation profiles and there exists some scientific evidence about the variability of bryndza which is lacking in connection to common characteristics of this Slovak cheese **(Šaková et al., 2015)**. Ewe´s cheese represents a matrix with a specific composition which reflected ewe´s milk and also by different autochthonous lactic acid bacteria (LAB) produce typical aroma profile of ewe´s lump cheese, barreled ewe´s cheese as well as bryndza cheese **(Šaková et al., 2015; Sádecká et al., 2016)**. **Kačániová et al. (2019)** identified 870 isolates from coliforms, enterococci, lactic acid bacteria, and yeasts in Slovak bryndza cheese by MALDI-TOF MS profiling. *Hafnia alvei*, *Klebsiella oxytoca*, *Lactococcus lactis*, and *Lactobacillus paracasei* were the most frequently identified species of bacteria. LAB group was represented by *Lactobacillus*, *Lactococcus,* and *Pediococcus*.

Several volatile organic compounds (VOC) of cheese, including raw milk-based ewe´s cheese, are formed by proteolysis and by the subsequent transformation of amino acids **(Ozturkoglu-Budak et al., 2016)** to α-keto acids **(Čaplová et al., 2018)**. Two different major pathways of amino acid degradation have been identified in *Lactococcus lactis* **(Yvon and Rijnen, 2001)**. The first pathway is initiated by an elimination reaction of methionine catalyzed by amino acid lyases and leads to major sulphur aroma compounds **(Dias and Weimer, 1998a; Dias and Weimer, 1998b)**. The second pathway is initiated by a transamination reaction catalyzed by aminotransferases, and has been observed especially for aromatic amino acids, branched chain amino acids, and methionine **(Rijnen et al., 1999; Bourdat-Deschamps et**

al., 2004). The resulting α-ketoacids are then degraded to aldehydes, alcohols, carboxylic acids, esters, methanethiol, and other sulphur compounds. Most of these compounds are produced by enzymatic degradation but a few ones result from chemical degradation in particular oxidation **(Nierop-Groot and de Bont, 1998, Nierop-Groot and de Bont, 1999)**. VOC are usually analyzed by gas chromatography after the extraction or pre-concentration of the volatile fraction. The most exhaustive methods for this purpose are high vacuum distillation (HVT), solvent-assisted flavour evaporation (SAFE), or solid phase microextraction (SPME) **(Sádecká et al., 2014)** combined with headspace. **Sádecká et al. (2014)** used SPME with gas chromatography-olfactometry (GC-O) for the determination of volatile odorants in May bryndza cheese. Depending on the degree of cheese maturation, from a GC-O point of view, 25 olfactometric responses from groups of alcohols, aldehydes, esters, ketones, fatty acids, and hydrocarbons were recorded.

The aim of this study was to obtain, for the first time, parallel information of principal volatile organic compounds in bryndza cheese determined by an electronic nose and a gas chromatography mass spectrometry with head-space solid phase microextraction sample pretreatment and to confirm the possibility of the use of e-nose for monitoring bryndza cheese quality.

Scientific hypothesis

Hypothesis 1: The impact of the season on bryndza cheese aroma profile determined by e-nose.

Hypothesis 2: The impact of the season on bryndza cheese aroma profile determined by HS-SPME-GC-MS.

Hypothesis 3: The application of the method to monitor bryndza cheese evaluation.

MATERIAL AND METHODOLOGY

Bryndza cheese samples

Samples of bryndza cheese were provided by 2 different producers. The first sample was produced from unpasteurized ewe´s milk by farm dairy. The second one was produced from a mixture of raw ewe´s (min. 50%) and pasteurized cow´s milk by industrial dairy. Samples were collected from May to September 2019. All samples (10) were places in sterile sample containers and transported to the laboratory on ice. Fresh samples were analyzed by head-space solid phase microextraction gas chromatography mass spectrometry (HS-SPME GC-MS) and electronic nose (e-nose) within one day after the delivery.

E-nose analysis

The electronic nose method (e-nose Heracles II, Alpha M.O.S., Toulouse, France) previously described by **Štefániková et al. (2019)** was used for sample analysis. For each analysis, 2.5 g of sample was incubated statically in a 20 mL vial in a thermostat block at 50 °C for 15 min (Autosampler, Alpha M.O.S.) and 5 mL volume of the headspace gaseous compounds was withdrawn using a headspace autosampler syringe and dispensed into the e-nose injector. The identification of compounds was performed by matching the measured peaks with Kovats´ retention indices with the NIST® library (The National Institute of Standards and Technology library) (>50%) by software Alpha Soft V14 (Alpha M.O.S.).

HP-SPME-GC-MS analysis

The head-space solid phase microextraction method was used for a sample extraction according to **Sádecká et al. (2014)** in a modified version. For each analysis, 2.5 g of sample was incubated statically in a 20 mL vial in a thermostat block at 50 °C for 30 min (CombiPal Autosampler 120, CTC Analytics AG, Zwingen, Switzerland), with an SPME fibre (1 cm; DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA) placed in the CombiPal.

Semi-quantitative composition of samples was determined by gas chromatography coupled with mass spectrometry (GC-MS) using an Agilent 7890B oven coupled with Agilent 5977A mass detector (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with column DB-WAXms (30 m × 0.32 mm × 0.25 μm; Agilent Technologies) operating with a temperature program and MS conditions according to **Sádecká et al. (2014)**.

The identification of compounds was carried out by comparison of mass spectra (over 80% match) with the NIST® 2017 library and retention times of reference standards (ethyl acetate, hexanoic acid, and isopentanol). The semi-quantitative content of determining compounds was calculated by dividing the individual peak area by total area of all peaks. Peaks under 1 % were not counted.

Statistical analysis

Compounds identified by e-nose with a discriminant >0.900 were selected, based on which the semi-qualitative evaluation was performed and PC analysis (Principal Component Analysis) was made by Alpha Soft V14 (Alpha M.O.S.) software. Descriptors were analyzed using single factor analysis of variance and significance was at $p < 0.05$.

The STATGRAPHICS Centurion (© StatPoint Technologies, Inc., USA) and GraphPad Prism 6.01 (GraphPad Software Incorporated, San Diego, California, USA) were used for statistical GC-MS analysis. The ANOVA method complemented by the Test of Tukey´s Multiple Comparison Test with a value of $p < 0.05$ was applied.

RESULTS AND DISCUSSION

In this study, the aromatic profiles of ten bryndza cheese samples by e-nose and HS-SPME GC-MS were evaluated. Bryndza cheese samples were collected from the Slovak dairies. Bryndza cheese is a soft spreadable cheese, made from unpasteurized ewe´s milk or a mixture of ewe´s and cow´s milk. **Kačániová et al. (2019)** previously described the microbiota studies of ewe´s bryndza cheese from the same mountainous regions of Slovakia. LAB, enterococci, and yeasts *Galactomyces candidus* play a key role in flavor development during cheese ripening **(Kačániová et al., 2020; Pangallo et al., 2014; Sádecká et al., 2019)**. The VOC´s are generated by the enzymatic degradation of amino acids in cheese, especially in cheese containing the only LAB.

Table 1 Determination and identification of volatile organic compounds with a discriminant >0.900 in bryndza cheese samples by e-nose.

	Compounds		Compounds
1	ethyl acetate	**14**	benzaldehyde
2	isopropyl acetate	**15**	acetaldehyde
3	isopentyl acetate	**16**	furfural
4	butyl acetate	**17**	2,3-butandione
5	ethyl propanoate	**18**	2-pentanone
6	ethyl butyrate	**19**	2-butanone
7	acetic acid	**20**	benzyl alcohol
8	butanoic acid	**21**	n-butanol
9	propanoic acid	**22**	2-propanol
10	propanal	**23**	1-hexanol
11	butanal	**24**	2-methyl propanol
12	3-methyl butanal	**25**	α-pinene
13	2-methyl propanal		

The amino acid transamination is catalyzed by lactococci aminotransferases and it is the first step in the degradation of aromatic and branches-chain amino acids which are precursors of aroma compounds **(Yvon et al., 1997; Tanous et al., 2005)**. The resulting α-ketoacids are then degraded to aldehydes, alcohols, fatty acids, esters, methanethiol, and other sulphur compounds **(Savijoki, Ingmer and Varmanen, 2006)**.

The identification of the compounds determined by e-nose was performed by matching the measured peaks with *Kovats´* retention indices with the NIST library. Aroma compounds identified in bryndza cheeses by e-nose are shown in Table 1. Total, 25 compounds with a discriminant >0.900 from class alcohols, esters, fatty acids, terpenes ketones, and aldehydes were identified. Ten compounds – 3-methyl butanal, 2-methyl-1-propanol, 2-butanone, 2-pentanone, ethyl acetate, isopentyl acetate, ethyl butyrate, acetic acid, butanoic acid, and butane-2,3-dione were identified in this study by e-nose and by gas chromatography-olfactometry previously described in bryndza cheese from Slovakia by **Sádecká et al. (2014, 2016, 2019)** and **Šaková et al. (2015)**.

Identified compounds by e-nose with a discriminant >0.900 were selected, based on which the semi-qualitative evaluation was performed by the principal component analysis (PCA). Figure 1 displays result processed by the PCA technique of the aroma profile of bryndza cheese samples. The first dimension (PC1 86.927%) allows the separation of July2 and August2 (positive score) from the other samples of cheese (negative score). Among samples May1, July2 and September2 (negative score of the second dimension PC2; 9.402%) and other samples of cheese (positive score PC2) statistically significant differences (p <0.05) were evident in their aroma profiles.

We confirmed differences in aroma profiles between May bryndza and summer or winter bryndza produced from ewe´s milk by the first dairy. On the contrary, more aroma profile differences were in bryndza cheese produced from the mixture of ewe´s and cow´s milk by the second dairy.

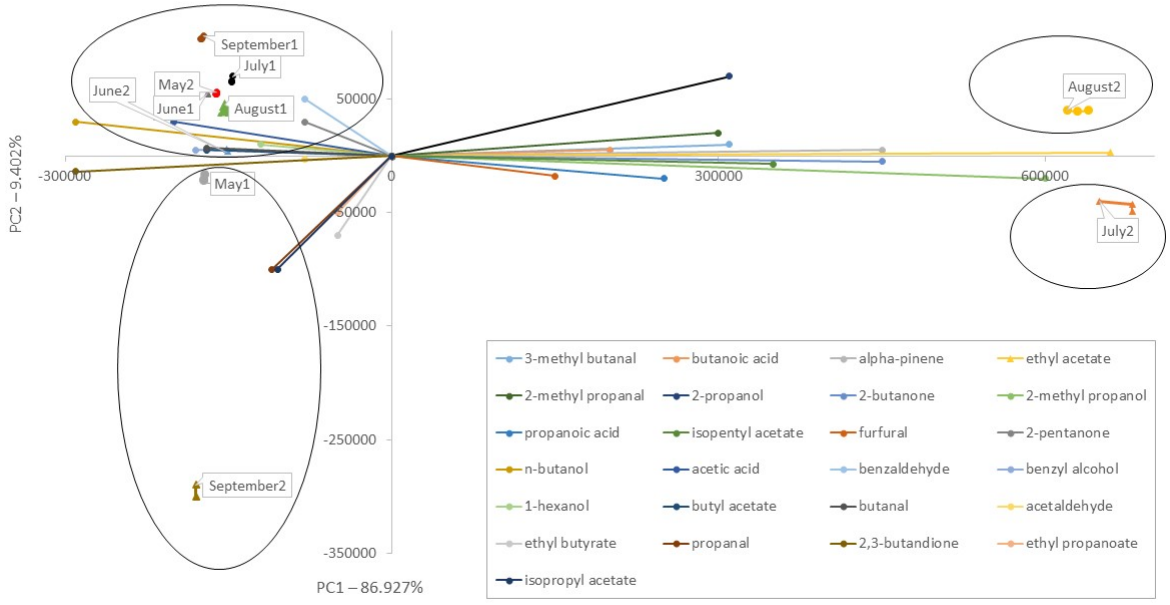

Figure 1 PCA analysis of the aromatic profile of the bryndza cheeses acquired by e-nose.

The samples collected in May and June showed no significant differences in aroma profiles when compared to each other but the aroma profiles were significantly different in comparison with July, August, and September samples and at the same time, the samples collected from July, August, and September showed statistically different (p <0.05) aroma profiles compared to each other.

On the contrary, the position of May2, June2, June1 – September1 samples on the negative score of axis 1 and a positive score of axis 2 could be explained by its higher proportions of acetic acid, benzyl alcohol, butyl acetate, butanal, benzaldehyde, n-butanol, 2-pentanone, and hexanol. The August2 sample is positioned at the opposite on axis 1 due to its higher proportions in butanoic acid, 2-propanol, 2-methyl propanal, 3-methyl butanal, α-pinene, and ethyl acetate. The sample of July2, positioned on the positive score of axis 1 and the negative score of axis 2, contains higher proportions of isopentyl acetate, 2-butanone, 2-methyl propanol, furfural, and propanoic acid and the position of May1 and September2 samples on the negative scores of axes 1 and 2 could be explained by their higher proportions of ethyl butyrate, ethyl propanoate, propanal, 2,3-butandione, and acetaldehyde.

Semi-quantitative content of identified volatile organic compounds in bryndza cheese samples determined by GC-MS is shown in Table 2. In total, 6 higher alcohols, 3 esters, 5 fatty acids, 3 ketones, and methoxy-phenyl-oxime were determined in the samples. Findings showed that principal odorants detected in all samples were acetoin (3.88% – 27.1 %), acetic acid (1.77% – 18.7%), dimethyl-silanediol (1.07% – 6.93%), methoxy-phenyl-oxime (1.78% – 7.01%), and isopentanol (1.72% – 7.44%).

The bryndza cheese from the first dairy contains about 15% of acetoin collected from May and June, and, on the contrary, the next months its content decreased (10.6%, 7.04%, and 10.5% respectively). The content of acetoin in samples from the second dairy was more variable, its amount was decreased (3.88%) in samples collected from July production and increased in samples collected from August production (27.1%).

Table 2 Semi-quantitative content (area %*) of identified volatile organic compounds in bryndza cheeses produced from ewe´s milk (1 – first dairy) and from mixture with cow´s milk (2 – second dairy) collected from May – September determined by GC-MS.

	Compounds	Bryndza cheese samples									
		May1	May2	June1	June2	July1	July2	August1	August2	September1	September2
		Area %									
1	ethyl acetate	1.94	2.62		4.65			10.27			
2	2-phenethyl acetate	0.95	1.08					16.6	0.62		
3	isopentyl acetate							1.76			
4	2-butanone[b]						16.2		16.8		
5	2,3-butanedione (diacetyl)		1.55						2.35	3.55	
6	3-hydroxy-2-butanone (acetoin)	15.2	16.8	15.7	18.5	10.6	3.88	7.04	27.1	10.5	9.73
7	acetic acid[a]	9.00	9.13	11.0	8.55	9.69	13.3	1.77	6.92	18.0	18.7
8	butanoic acid[a]	1.89	1.84	2.21	2.03	1.91	2.11		1.22		
9	pentanoic acid[b]	0.73						1.28			
10	hexanoic acid[a]	3.92	4.07	3.19	2.00	4.10	3.10		2.22		
11	octanoic acid[a]	1.51	1.37			2.42					
12	2-butanol						12.4				
13	2,3-butanediol[a]	3.83	3.67			2.41		1.54		5.42	4.21
14	2,7-dimethyl-4,5-octanediol[a]	1.01	1.06								
15	dimethyl-silanediol[a]	3.99	3.98	2.17	2.3	3.03	2.48	1.07	2.34	6.93	6.16
16	3-methyl-1-butanol (isopentanol)	5.03	4.69	2.06	2.82	3.41	3.21	7.44	1.72	2.41	3.68
17	2-phenyl ethanol	1.05	1.32					12.40	0.61		
18	methoxy-phenyl-oxime[a]	5.13	4.54	7.1	7.01	5.48	5.03	1.87	3.29	2.44	1.78

Note: *listed are the components that represented min. 1% in at least one bryndza cheese sample. Letters in superscript indicates statistically significant difference: a – among samples depending on month of production, b – among samples depending on kind of milk.

The content of acetic acid was in a range of 8.55% – 13.3% in samples produced in May-July, it was decreased in August (1.77% and 6.92%, respectively) and increased in September (18.0% and 18.7%, respectively). While acetoin and acetic acid were the most representative compounds in samples produced in May and June, samples produced in July and August had different profiles. While the samples from the first dairy had a higher content of acetoin and acetic acid (July) and 2-phenethyl acetate and 2-phenyl ethanol (August), the samples from the second dairy had a higher content of 2-butanone and acetic acid (July) and 2-butanone and acetoin (August). Bryndza cheese produced in September had a weaker aroma, but acetic acid and acetoin were identified in the higher amount than in the May and June samples. Statistical analysis by the Test of Tukey´s Multiple Comparison Test confirmed that the amount of eight aromatic compounds (Table 2) was influenced by the month of production. On the other hand, only two aroma substances were notably influenced by the kind of milk.

The compounds identified by the electronic nose with a discriminant >0.900 with ethyl acetate, isopentyl acetate, 2-butanone, acetic and butanoic acids, and butane-2,3-dione were confirmed by GC-MS. Several of the identified compounds (ethyl acetate, 2-phenylethyl acetate, ethyl propanoate, 2-methyl-propanol, 2-phenyl ethanol, 2,3-butanediol, 3-hydroxy-2-butanone (acetoin), 3-methyl butanol, 2,3-butanedione, acetic, butanoic, pentanoic, and octanoic acids) are known to be components of different foreign ewe´s cheese such as the Oscypek, Canestrato Pugliese, Fiore Sardo, Torta del Casar, Terrincho, Roncal, Manchego, and Pecorino Romano **(Barron et al., 2005; Massouras, Pappa and Mallatou, 2006; Majcher et al., 2011; Sádecká et al., 2014; Delgado-Martínez et al., 2019)**.

Other identified compounds, benzaldehyde **(Bourdat-Deschamps et al., 2004)**, 2-pentanone **(Gallegos et al., 2017)**, hexanal **(Gómez-Torres et al., 2016)**, 2,7-dimethyl-4,5-octanediol **(Nájera-Domínguez et al., 2015)** were previously described as secondary metabolites by LAB. **Passerini et al. (2013)** confirmed that strains of *L. lactis* with the *cit*P gene and the *cit*M-G cluster produced a larger amount of aroma-active compounds than the strains without this genetic information. **Bozoudi et al. (2018)** and **Iussig et al. (2015)** reported the quality differences of milk and dairy products from different grazing areas. The terpenoid composition (limonene, myrcene, carvone) of milk and cheese are directly transferred from ingested botanical species and free fatty acids (from acetic to dodecanoic acid) can also be effective to trace animal management and feeding systems **(Moran et al., 2019)**. The free fatty acids are also precursors of methyl ketones, alcohols, lactones, and esters, so they may play an important role in the global aroma development of cheese **(Delgado-Martínez et al., 2019)**. Fatty acids were the most abundant VOCs in the barrelled ewes' types of cheese (intermediate product in the production of winter bryndza) from Slovakia **(Sádecká et al., 2016)** and in the raw ewe´s milk cheese, Feta cheese and Torta del Casar **(Bozoudi et al., 2018; Delgado et al., 2010; Delgado-Martínez et al., 2019)**. The identified 2-methyl propanal **(Griffiths, 2010)** was previously described as milk aromas. The other compounds, n-butanol, dimethyl-silanediol, and methoxy-phenyl-oxime were identified in cheeses produced from pasteurization milk fermented with mixed cultures (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) and with *Dregea sinensis* Hemsl. protease **(Wang, Wang and Huang, 2017)**.

The e-nose technology in this study can detect the fingerprint of volatile organic compounds present in the headspace of the bryndza sample and parenica cheese previously described **(Štefániková et al., 2019)** by the means of a gas chromatography principle. Several studies used an e-nose method containing 10 metal-oxide semiconductors for characterization of the aroma profile of French cheese types, Danish blue cheese, or Pecorino cheese **(Ghasemi-Varnamkhasti et al., 2019; Trihaas, Vognsen and Nielsen, 2005; Cevoli et al., 2011)**. There was used the e-nose with sensors in the above-mentioned studies, which could not determine or identify concrete volatile organic compounds and therefore there was a need to confirm the results by GC methods.

CONCLUSION

This study has proved for the first time the possibility of bryndza cheese quality evaluation using an e-nose with GC columns and FID detectors. The results were compared with gas chromatography with mass spectrometry. The e-nose method can determine the aroma profile of samples in a short time and the results may be supplemented by aensory evaluation by the assessors. The e-nose may take great advantages over GC-MS in distinguishing the integral aroma profiles, although it cannot identify the explicit volatile compounds of different samples.

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INFLUENCE OF INULIN AND OLIGOFRUCTOSE ON THE SENSORY PROPERTIES AND ANTIOXIDANT ACTIVITY OF APPLE JELLY

Mariusz Witczak, Grażyna Jaworska, Teresa Witczak

ABSTRACT
The objective of this study was to analyse the influence of inulin with different degrees of polymerization (DP values) and oligofructose preparation on the sensory properties and antioxidant activity of apple jelly. It has been determined that both the addition of inulin (independently of DP) as well as oligofructose significantly modifies colour and sensory properties and influence antioxidant activity of apple jelly. It has been observed that the manner in which water is bound by the applied preparations may have a significant impact on the analysed properties of jelly. In terms of taste, the highest scores were awarded to the desserts with addition of long-chain inulin and in terms of the overall sensory evaluation those with addition of preparation with medium length chain. The variability of the sensory properties depended on the type and level of the additive, and this impact varied between individual preparation types. The conducted study has enabled the conclusion that inulin may pose an attractive ingredient of desserts with health-promoting properties.

Keywords: apple; inulin, antioxidant; activity; sensory

INTRODUCTION
Inulin is a natural plant biopolymer that belongs to the group of long chain fructans. In plants, inulin acts as a long-term reserve material accumulates mainly in underground parts of plants or short-term in places of active growth. Inulin is stored in largest quantities in the roots and tubers of the *Asteraceae* plant (Jerusalem artichoke, dahlia, artichoke, burdock, asparagus, dandelion) and of the lily (onion, leek, garlic). In smaller amounts inulin occurs in the overground parts of the *Poaceae* plants (rye, wheat, triticale, barley). The most important source of inulin for an industrial scale are Jerusalem artichoke and chicory **(Kiełtyka-Dadasiewicz et al., 2014; Meyer et al., 2011; Nowak et al., 2012; Śliżewska et al., 2013; Trabs, Kasprick and Henle, 2011; Witczak, Jaworska and Witczak, 2020)**.

Due to the fact that inulin has very good technological properties and has an positive health-promoting effect on the human body, it is gaining more and more recognition among both food producers and consumers. Particular attention is paid to the prebiotic and dietary properties of inulin as well as its ability to improve the sensory characteristics of the product. For these reasons inulin is largely used in food production as a texture modifier, substitute of fat and sugar **(Florowska and Krygier, 2007)**.

Inulin properties depend on the source of inulin that affects polymerization degree and the nature of the bonds **(Glibowski, Kulik and Masternak, 2012; Guimarães et al., 2018)**. Physical and chemical properties of inulin (gelation and swelling ability, ability to emulsion formation, thickening, structure stabilization) are widely utilized in the food industry. In the dairy industry, it is used for the production of yoghurts and dairy desserts, in the fruit and vegetable industry for the production of juices, jams, in the fat industry for the production of margarines, in the meat industry for the production of sausages and in the confectionery industry for the production of cookies **(Delgado and Bañón, 2015; Esmaeilnejad Moghadam et al., 2019; Rodriguez Furlán and Campderrós, 2017; Keenan et al., 2014; Rodríguez-García, Sahi and Hernando, 2014; Tárrega, Torres and Costell, 2011; Witczak, Witczak and Ziobro, 2014; Ziobro et al., 2013)**. However, data on the use of inulin in dessert products are limited and they mainly concern milk-based desserts **(Esmaeilnejad Moghadam et al., 2019; Rodriguez Furlán and Campderrós, 2017; Tárrega, Torres and Costell, 2011)**.

The present study aimed at determining the influence of commercial products based on inulin at various polymerization degrees and oligofructose preparation on the antioxidant activity and sensory evaluation of apple jelly.

Scientific hypothesis

Inulin may constitute an attractive alternative for the development of fruit dessert recipes with good sensory and prebiotic properties.

MATERIAL AND METHODOLOGY

Materials

The material used in formulations for jelly preparation consisted of an apple concentrate with the extract content of 69.5% (Apkon Sp. z o.o, Przemyśl, Poland), natural apple flavor (100-fold concentrated), HPX and GR inulin preparations (Beneo, Orafti, Belgium) with different average DP, oligofructose P95 (Beneo, Orafti, Belgium), pectin (Pektowin, Poland). According to the product specification inulin GR contains additionally of a mixture of glucose, fructose and sucrose (8%), HPX 100% of inulin and the P95 preparation contains 95% oligofructose.

Sample preparation

The sample preparation was described in the work of **Witczak, Jaworska and Witczak (2020)**. In brief: the mixture of apple concentrate with water and sugar was boiled, inulin or oligofructose preparation was added, pectin was added, and the mixture was mixed, with citric acid and apple flavour being subsequently added. The solutions were poured hot into jars and were subject to pasteurization at 80 °C ±2 °C for 10 minutes. The obtained samples were cooled down and stored in refrigeration conditions (4 – 6 °C). Analyses were performed on the first day and after 6 weeks of storage. Control jelly was based on the following ingredients (per 1 kg): apple concentrate 152 g, sugar 192 g, pectin 9 g, citric acid 6 g, apple aroma 10 mL, water 631 mL. In samples 3% 30 mL of water was replaced with 30 g, and in 6% 50 mL of water with 60 g of the studied preparations.

Methods

Colour A_{420} was determined via **Burdulu and Karadeniz (2003)** method with own modification for aqueous extracts, prepared via mixing 1 g of jelly with 7 mL of distilled water. The whole mixture was stirred until the jelly dissolved in water entirely. Absorbance measurement was performed on the UV-160A spectrophotometer (Shimadzu, Japan) at 420 nm wavelength.

Total polyphenols and antioxidant activity was determined in methanol extracts. Spectrophotometry with the Folin-Ciocalteu reagent was used for the measurement of the total polyphenol content. Absorbance was measured on the UV-160A Spectrophotometer (Shimadzu, Japan) at wavelength 675 nm against 80% methanol. Polyphenols content was read from the standard curve for (+) – catechin **(Singleton, Orthofer, Lamuela-Raventós, 1999)**.

Antioxidant activity was determined with the use of free radicals 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) according to the method proposed by **Re et al. (1999)**. Absorbance measurement was performed after 10 minutes, using the UV-160A spectrophotometer (Shimadzu, Japan) at wavelength of 734 nm. Antioxidant activity was expressed as mmol TE per 100 g d.w. (Trolox Equivalent).

Antioxidative activity was determined with the FRAP method according to the **Benzie and Strain (1996)** method. Samples were incubated with acetic buffer, $FeCl_3$ and TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) solution for 10 minutes and subsequently the absorbance was measured (UV-160A spectrophotometer, SHIMADZU, Japan) at wavelength 595 nm against 80% methanol. The value of antioxidative activity was expressed in μmol Fe^{2+} per g of the edible parts of the jelly.

The sensory evaluation was performed using 5-point scale (5 = excellent, 4 = very good, 3 = good, 2 = bad, 1 = very bad). 8 persons with verified sensory sensitivity participated in the testing. Quality markers such as: surface and clarity, syneresis, colour, consistency, smell and taste. These markers corresponded to importance coefficients, respectively: 2, 2, 4, 4, 4, 4. Selection of importance coefficients was performed based on a discussion conducted within the evaluating panel prior to the evaluation.

Statistical analysis

The data were treated by one-factor analysis of variance (ANOVA), and the averages were compared with the using Duncan test at significance level 0.05. The influence of selected factors was analysed with the use of two- or three- factorial analysis of variance. Additionally the values of Pearson's correlation coefficients between selected parameters characterizing samples properties were calculated. All calculations were performed with statistical software package Statistica 12.0 (StatSoft Inc., USA).

RESULTS AND DISCUSSION

Colour characteristics

Colour measured by means of spectrometry (Table 1) after preparation exhibited the lowest value for samples with 3% P95 addition and the highest for samples with 6% addition of HPX. Samples with addition of inulin with the highest degree of polymerization at lower concentration did not differ from control sample, and at a higher concentration (6%) they were characterized by significantly higher intensity ($p = 0.035$). At the moderate degree of polymerization (GR preparation), samples at both concentrations did not differ significantly from control sample, whereas lower degree of polymerization (P95 oligofructose) resulted in reduced colour intensity with lower concentration, followed by its increase. However, in both cases the obtained values were lower than in control sample. It should be noted that according to the results of the multi-factor variance analysis, all analysed values (time, level and type of additive) had significant impact on the A420 colour. However, as it has been determined, after 6 weeks of storage, in contrast to the first day of storage, no differences could be found between the samples, yet they were also characterized by greater variability. The small variability of the A_{420} parameter obtained in this work coincides with the results obtained earlier **(Morreale, Benavent-Gil, Rosell, 2019; Witczak, Jaworska and Witczak, 2020)**.

Table 1 Color, total polyphenols and antioxidant properties of jellies.

Sample	Week	Colour A_{420}	Total polyphenols	ABTS*	FRAP
		nm	mg·(100 g)$^{-1}$	(μM Trolox.g^{-1})	(μM Fe^{2+}.g^{-1})
Control	**0**	0.234 ±0.013^{bc}	16.9 ±0.4^{cd}	20.2 ±2.2^{bcd}	3.27 ±0.20
	6	0.727 ±0.006^{e}	3.43 ±0.5^{ab}	15.9 ±1.6^{a}	3.39 ±0.03
3% HPX	**0**	0.234 ±0.003^{bc}	17.1 ±1.0^{cd}	20.2 ±1.5^{bcd}	3.19 ±0.20
	6	0.734 ±0.007^{e}	4.20 ±0.4^{b}	18.6 ±1.9^{ab}	3.51 ±0.20
6% HPX	**0**	0.330 ±0.023^{d}	17.1 ±1.0^{cd}	21.1 ±1.8^{bcd}	3.01 ±0.46
	6	0.718 ±0.023^{e}	3.71 ±0.4^{b}	18.3 ±1.5^{ab}	3.44 ±0.05
3% GR	**0**	0.236 ±0.000^{bc}	15.8 ±0. 8^{c}	21.9 ±2.4^{cde}	3.48 ±0.16
	6	0.727 ±0.004^{e}	2.27 ±0.2^{a}	19.7 ±1.7^{bc}	3.24 ±0.15
6% GR	**0**	0.249 ±0.001^{c}	15.9 ±0.8^{c}	23.0 ±1.9^{de}	3.41 ±0.24
	6	0.711 ±0.007^{e}	4.75 ±0.8^{b}	19.6 ±1.2^{bc}	3.38 ±0.27
3% P95	**0**	0.188 ±0.003^{a}	17.1 ±1.0^{cd}	24.5 ±1.4^{e}	3.34 ±0.33
	6	0.711 ±0.022^{e}	3.61 ±0.6^{b}	21.0 ±0.8^{bcd}	2.95 ±0.29
6% P95	**0**	0.220 ±0.016^{b}	17.9 ±0.6^{d}	23.0 ±1.1^{de}	3.43 ±0.31
	6	0.725 ±0.001^{e}	3.76 ±1.0^{b}	18.9 ±1.0^{bc}	3.22 ±0.24
One-way ANOVA – *p*		<0.001	<0.001	<0.001	0.221
Three-way ANOVA – *p*					
Factor A (type)		<0.001	0.005	0.005	0.376
Factor B (level)		0.001	0.041	0.524	0.712
Factor C (time)		<0.001	<0.001	0.000	0.983
Factor A× Factor B		0.015	0.038	0.189	0.339
Factor A× Factor C		<0.001	0.052	0.492	0.006
Factor B× Factor C		<0.001	0.409	0.363	0.318
A× B× C		0.003	0.022	0.967	0.973

Note: Mean value of three replication ± standard deviation. Mean values signed this same letters in particular columns are non-significant different at 0.05 level of confidence.

Table 2 Sensory evaluation of jellies.

Sample	Sensory evaluation					
	Surface and clarity	Color	Consistency	Smell	Taste	Overall score
Control	5.0 ±0.0	5.0 ±0.1^{c}	3.9 ±0.2^{a}	5.0 ±0.0^{d}	4.4 ±0.4	4.5 ±0.5^{ab}
3% HPX	3.7 ±0.9	4.5 ±0.4^{ab}	4.7 ±0.3^{b}	4.2 ±0.5^{ab}	4.8 ±0.2	4.5 ±0.1^{a}
6% HPX	3.7 ±0.9	4.6 ±0.2^{abc}	4.4 ±0.5^{b}	3.9 ±0.1^{a}	4.6 ±0.2	4.4 ±0.1^{a}
3% GR	4.4 ±0.8	4.9 ±0.1^{bc}	4.8 ±0.3^{b}	4.7 ±0.2^{cd}	4.8 ±0.3	4.8 ±0.1^{b}
6% GR	4.4 ±0.8	4.7 ±0.5^{abc}	4.8 ±0.2^{b}	4.5 ±0.3^{bc}	4.7 ±0.2	4.7 ±0.1^{ab}
3% P95	4.4 ±0.4	4.4 ±0.4^{a}	4.4 ±0.5^{b}	4.1 ±0.2^{ab}	4.7 ±0.4	4.4 ±0.3^{a}
6% P95	4.5 ±0.4	4.5 ±0.4^{ab}	4.5 ±0.3^{b}	3.8 ±0.5^{a}	4.6 ±0.4	4.4 ±0.2^{a}
One-way ANOVA – *p*	0.055	0.041	0.007	<0.001	0.643	0.046
Two-way ANOVA – *p*						
Factor A (type)	0.034	0.036	0.122	0.000	0.699	0.005
Factor B (level)	0.895	0.972	0.617	0.024	0.312	0.217
Factor A× Factor B	0.982	0.535	0.539	0.957	0.971	0.844

Note: Mean value of five replication ± standard deviation. Mean values signed this same letters in particular columns are non-significant different at 0.05 level of confidence.

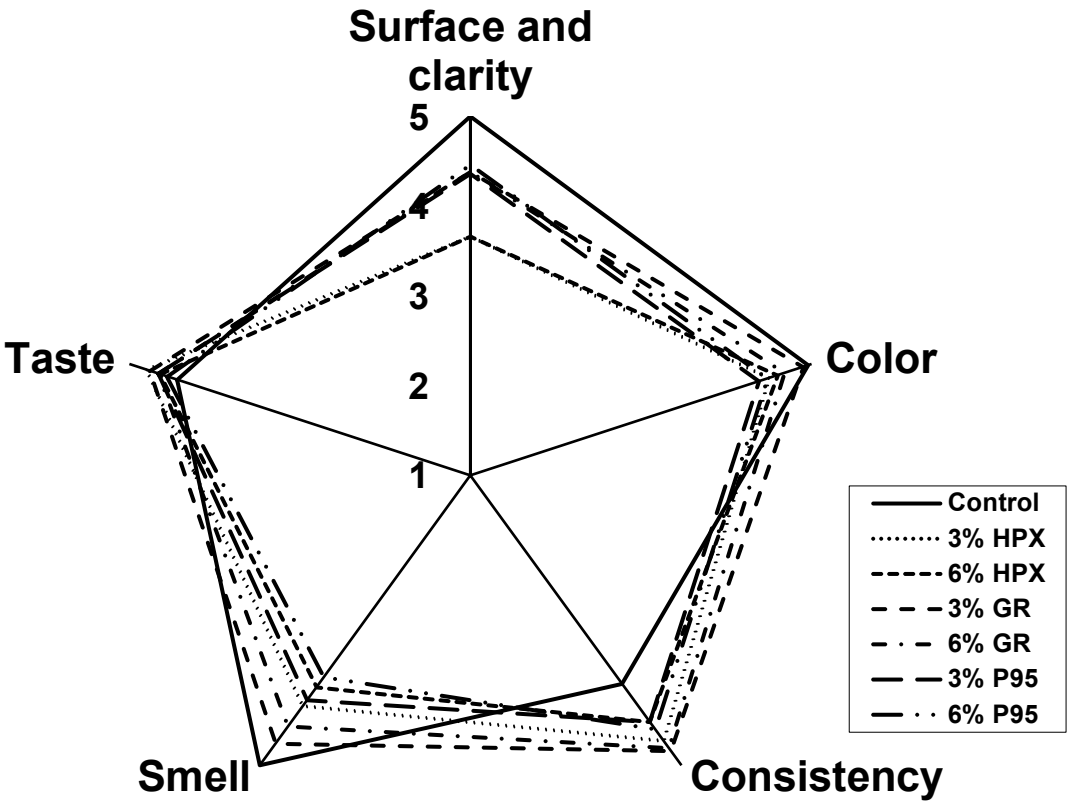

Figure 1 Sensory evaluation of jellies.

Morreale, Benavent-Gil and Rosell (2019) did not find a direct impact of DP on the values characterizing the color of gluten-free bread, but the authors found variability due to yeast type and inulin producer. In turn, **Witczak, Jaworska and Witczak (2020)** found the variability of the L* parameter responsible for the color lightness depending on the type and amount of inulin added. The analysis of the cited works and the results obtained in this work allows us to state that the change in color is conditioned by inulin interactions with the other components of the system, and the variability is not directly related to inulin DP.

Polyphenol content

On the first day of storage only samples with the addition of GR preparation were characterized by lowered content of total polyphenols (Table 1) relative to the control sample, however, also in this case statistically significant differences could not be found. Slightly larger reduction (ca. 10%) in polyphenol content in orange jellie was found after adding trehalose **(Kopjar et al., 2016)**. In turn, **Cano-Lamadrid et al. (2020)** found significant changes in polyphenol content depending on the composition of jelly candies based on pomegranate juice. The authors **(Cano-Lamadrid et al., 2020)** found a decrease in the content of polyphenols with an increase in the addition of gelatin and an increase with the replacement of sugar with honey.

Similarly as after preparation, following the six week storage period, no significant differentiation relative to control sample could be determined. However, in the stored samples, a clearly differentiated impact of individual preparations and concentration levels can be seen. However, a statistically significant change with increased concentration was determined only in the case of the GR preparation (p = 0.006). Similar effect of limiting the decline in polyphenol content during storage was found for orange jellie with the addition of trehalose, what according to the authors is due to the protective effect of the additives used **(Kopjar et al., 2016)**.

Antioxidant activity

An increase of antioxidant activity determined via ABTS method was found (Table 1). The preparation with the longest chain produced a minor increase, while the oligofructose preparation P95 changed the value of ABTS activity to the largest extent. However, statistically significant differences relative to control sample were determined only in the case of 3% addition of this preparation. Similar increase in antioxidant activity determined via ABTS method was found after introducing trehalose into orange jelly and depending on the addition of gelatin for jelly candies based on pomegranate juice **(Cano-Lamadrid et al., 2020; Kopjar et al., 2016)**. After the six week storage period, the samples with addition of GR and P95 preparations were characterized by significantly higher values of ABTS activity than control sample. Samples with addition of the HPX preparation did not differ statistically, although also in this case the values were higher than in control sample. Similar relationship

after storage was obtained for the addition of trehalose to orange jelly **(Kopjar et al., 2016)**. According to **Kopjar et al. (2016)** the change in antioxidant activity may be caused by the degradation of the present antioxidants or their chemical modification.

No statistically significant differentiation of activity determined via FRAP method could be found (Table 1). However, also in this case certain trends can be observed. After preparation, lower values were recorded for samples with HPX addition, in the two remaining cases the values were higher than for control sample. These values differed slightly after storage, where the lowest FRAP activity value was determined for samples with P95 addition. The difference of the behavior of the antioxidant activity depending on the method is due to the fact that each method is specific to only one antioxidant mechanism **(Cano-Lamadrid et al., 2020)**.

Sensory evaluation

Figure 1 and Table 2 presents results of sensory evaluation of the tested jellies. In the case of surface and clarity, no statistically significant difference in one-factor variance analysis could be found. However, a two-factor variance analysis indicated a significant impact of the additive type. This is further indicated by the evaluation results. Control sample was awarded the highest scores, while the lowest to the sample with HPX addition at both levels. The two remaining preparations produced jellies with a similar evaluations, yet significantly lower than control. No differentiation was observed between samples in the case of syneresis (data unpublished). The analysed samples differed significantly in terms of colour (at the level of 0.05). Similarly to surface and clarity, the highest score was awarded to control sample. Similar scores were awarded to the sample with addition of 3% GR, and only slightly lower scores were awarded to samples with 6% addition of HPX and GR preparations – in these cases the differences were statistically insignificant relative to control sample. Worse scores were obtained for the remaining cases. Two-factor variance analysis demonstrated statistically significant impact of the type of preparation used, yet no impact of the addition level. According to the results of one-factor analysis of variance results, the samples differed significantly in terms of consistency and in this case the control sample was awarded the lowest scores. The highest scores were awarded to samples with GR addition (at both levels) and 3% HPX. Slightly lower scores, yet still better than for control sample, were awarded to samples with addition of P95 and 6% HPX. However, it should be noted that according to the results of statistical analyses, no significant differentiation between samples with addition of preparations occurred and only control sample differed significantly from the remaining samples. The greatest differentiation was obtained in the case of smell. In this case, statistical analysis indicates a significant impact of both type and level of additive. The highest score was awarded to control sample and value of the score decreased with the increase of addition level, independently of preparation type. Although samples with P95 addition were awarded the lowest scores, this evaluation was very close to the scores obtained for HPX (statistically insignificant differences). No statistically significant differences were obtained for taste evaluation. However, analysis of the results shows that control sample was awarded the lowest score. The remaining samples were assessed at a very similar level, and the best scores were awarded to samples with addition of 3% inulin products (HPX and GR). The overall evaluation enables the statement that the GR granulated inulin preparation has been evaluated most favourably among the analysed products. The two remaining products obtained overall evaluations at a similar level as control sample. It should be also noted that evaluations for different concentrations were identical or remained at a very similar level. This indicates that a suitably selected recipe stemming from the balance between the amount of preparation and water enables obtaining samples with highly similar sensory traits. Moreover, overall evaluation demonstrates the possibility of utilising all tested preparations as ingredients of jellies based on apple concentrate. These results comply with the results obtained for other products. Values comparable with control in terms of taste, smell and consistency were obtained for kefir with addition of inulin **(Glibowski and Kowalska, 2012)**. On the other hand, **Gramza-Michałowska and Górecka (2009)** obtained increased scores for point evaluations of products with addition of inulin – sponge cake, cake cream, natural yoghurt and ground pork burgers. The study of **Brennan and Tudorica (2008)** analysed the impact of the level and type of dietary fibre on yoghurt properties. No significant differentiation of the overall sensory evaluation was found, although the highest overall evaluation score was awarded to samples with 6% addition of inulin and 6% skimmed milk powder.

CONCLUSION

The obtained results have enabled the statement that addition of inulin and oligofructose significantly modifies the properties of apple jellies. Colour parameter A420 (corresponding to the colour lightness) were subject to a considerable variability. Sensory analysis has demonstrated strong impact of additives only on the smell characteristics. Despite the fact that significant differences have been shown, the variability of the remaining parameters of sensory characteristics was not high. It should be underlined that the highest scores in terms of taste were obtained for the jelly supplemented with HPX preparation, and in terms of overall evaluation with the samples with the addition of GR preparation. A significant impact of additives was observed on the total polyphenols content and antioxidant activity determined via the ABTS method. However, no variability could be found for activity determined via FRAP method. The conducted analyses provide an indication that inulin may constitute an attractive alternative for the development of fruit dessert recipes, which would enable obtaining products with prebiotic properties.

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OCCURRENCE OF RESISTANCE TO ANTIBIOTICS THERAPY IN COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM SHEEP´S MILK IN HOLDING IN SLOVAKIA

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ABSTRACT

The occurrence of bacteria *Staphylococcus* spp. was examined in a total of 3466 individuals and in 12 pool milk samples from 2017 to 2019. The experiment was carried out in two herds of the breed of sheep, Improved Valaska, in the Slovakia region. Eleven species of the genus *Staphylococcus* spp. (n = 431) were isolated and taxonomically identified. From the coagulase-positive staphylococci (CPS), *S. aureus* was isolated during the reporting period, however, most often in the third year (50). The incidence of *S. intermedius* and *S. hyicus* were irregular. The incidence of *S. schleiferi* was highest at the end of the follow-up duration. From the coagulase-negative staphylococci (CNS) (n = 158), were isolated *S. epidermidis* present in 20.4% (88) and *S. chromogenes* 11.4 % (49), *S. caprae, S. xylosus,* and other species rarely occurred. *S. aureus* (n = 133) showed maximum resistance to erythromycin 12.0%, novobiocin 10.5%, and neomycin 9.0%. The incidence of intermedial susceptibility was observed predominantly to a penicillin (16 strains), novobiocin (11 strains), erythromycin (14 strains), oxacillin, and cloxacillin (12 strains), neomycin (11 strains), and lincomycin (9 strains). Observantly, *S. schleiferi* (n = 101) showed the highest resistance to novobiocin (5.9%) and erythromycin (5.0%), however, a high incidence of intermediate susceptibility to erythromycin (9), amoxicillin, novobiocin (8), ampicillin, lincomycin (7), penicillin, methicilin and cefoperazone (5 strains) can be identified as adverse. The incidence of resistant and intermediate sensitive test strains *S. aureus* and *S. schleiferi*, especially for erythromycin, novobiocin, and neomycin, which are often used to treat udder inflammation in sheep, is relatively adverse.

Keywords: Sheep milk; coagulase-positive staphylococci; coagulase-negative staphylococci; antibiotics resistance; mastitis

INTRODUCTION

Mastitis is one of the most common diseases that affects dairy sheep. Mastitis leads to major economic loss, mainly due to discarded milk, reduced milk production, and quality, alteration of cheese-making properties, early culling, and increased health care costs **(Legarra et al., 2007)**.

Over 100 different microorganisms cause mastitis, particularly, coliform bacteria, staphylococci, and streptococci **(Smith and Hogan, 2001)**. In dairy sheep, the most important agents involved in clinical mastitis are bacterial infections, while the most frequently isolated pathogens are coagulase-negative staphylococci (CNS) present on and around the udder skin **(Leitner, Silanikove and Merin, 2008)**, with different pathogenicity causing clinical and subclinical mastitis **(Riggio and Portolano, 2015)**. Mastitis is of both extreme zoonotic and economic importance. It is the cause of multiple hazardous effects on human health and animal production.

Recently, it was recognized that the antimicrobial susceptibility of coagulase-negative staphylococci, which represents the majority of organisms isolated from ovine milk, is important for the early recognition of newly emerging resistant milk-borne bacterial agents **(Onni et al., 2011)**. Furthermore, with reference to specific conditions of ruminants, many authors in their works referred to these as the most frequently isolated: *S. epidermidis, S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. warneri,* and *S. sciuri* **(Fthenakis, 1994; Ergün et al., 2009)**.

Although, CNS does not have a comparable range for the virulence factors, such as *S. aureus*, one of the important factors of virulence is the ability to create resistance to an antibiotic, while some were described as multiresistant **(Moniri, Dastegholi, and Akramian, 2007)**. Multidrug resistance is defined as the resistance of three or more classes of antimicrobial agents **(Schwarz et al., 2010)**. The use of antibiotics is the most common treatment for these cases **(Gomes and Henriques, 2015)**, and β-lactams are the most frequently used classes for the treatment of mastitis. Additionally, mastitis therapy is usually started before the results of the antimicrobial susceptibility test of

pathogens **(Hendriksen et al., 2008)**, thus, representing one of the most important reasons for treatment failure. Moreover, this antibacterial strategy has many disadvantages including a low cure rate, increasing the presence of antibiotics residues, and the occurrence of antimicrobial resistance **(Minst et al., 2012)**. Mastitis resistance is a complex and multifactorial trait, and its expression depends on both genetic and environmental factors, including infection pressure **(Tolone et al., 2016)**. In the broadest sense, resistance can be defined as the ability to avoid any infection and/or the quick recovery from an infection **(Rupp and Boichard 2003; Rupp and Foucras, 2010)**. This involves different factors such as preventing the entry of the pathogen into the mammary gland, inducing an immune response capable of limiting pathogen development in the udder and recovery from the infection, as well as controlling the pathogenic effects of the infection, such as tissue damage **(Rupp et al., 2009)**. Resistance to antibiotics may be acquired by spontaneously occurring genetic mutations and, more commonly, by the horizontal transfer of mobile DNA elements from a donor cell to another bacterial species **(Chambers, 2001)**.

Over the years, extensive use of antimicrobials has led to increasingly resistant bacteria at an alarming rate and has become a serious concern worldwide. To ensure suitable antibiotic therapy, bacterial isolation, and evaluation of antibiotic susceptibility are essential. Also, milk produced from animals with subclinical mastitis poses serious veterinary and epidemiology risk since its rich nutrient composition and neutral pH make it a good vehicle for the survival and growth of bacteria. Resistant bacteria may contaminate food products, which could be transmitted to humans through the food chain. This underlines the importance of pathogen surveillance. Consequently, monitoring pathogens and their antimicrobial resistance patterns have become today's necessity **(Ceniti et al., 2017)**.

Scientific hypothesis

This work aimed to determine the occurrence and the most common types of staphylococci. Individual pools and sheep's milk samples were investigated, and a comparison of the incidences of antibiotic resistance of the most numerous tested species *S. aureus* and *S. schleiferi* was conducted.

MATERIALS AND METHODS

Characteristics of experimental breeds of sheep

One of the breeds of sheep with 350 Improved Valaska sheep and another farm with 280 sheep with a program of gradual crossing with the "Lacaune" breed were used for the experiment. Tracking the etiology in mastitis in the findings of the pool samples was carried out during the three seasons of the machine milking in the holdings, compliant with the technological standards of Slovakia. During the three seasons between April and September, a total of 12 complex examinations were repeatedly performed. A significant measure in the course of the experiment was to treat all cases of clinical mastitis solely based on proven susceptibility to a range of selected antibiotics.

Testing and sampling of the herds' sheep's milk

Clinical examination of the udder supplemented by a Californian Mastitis Test individual sheep's milk was conducted at the beginning and the end of each season, alongside a bacteriological examination of samples according to the principles posited by these authors **(Fthenakis, 1994; Vasil, 2004; Mørk et al., 2007)**. Emphasis was placed on aseptic sampling and transport of the mixed pools samples and the individual sheep's milk samples intended for bacteriological examination.

Bacteriological examination

The inoculum of each sample of milk was inoculated on the plates with 5% blood agar, incubated at 37 °C, and after 24 hours of reading. When the growth was more than 7 colonies from one type of colony, it was inoculated and cultured on selective nutrient soils. Identification of *Staphylococcus* spp. bacterial cultures were carried out by assessing the growth of suspected bacteria on nutrient agars (5% of blood Agar, N° 110, Baird-Parker agar, Brilliance UTI Clarity Agar (Oxoid Ltd., Basingstoke, Hants, UK). The pigment formation, hemolysis, catalase positivity, Gram positivity, creation of free or coupled coagulase, and other characters, were determined. The identification of each species was made by STAPHYtest 24 and evaluated by TNW ProAuto 7.0 (Erba-Lachema, Brno, Czech Republic) with a probability of correct designations of the kind above 90%. The functionality of the set was controlled using a strain of *Staphylococcus aureus* CCM 7113 (CCM, Masaryk University, Brno, Czech Republic).

Testing of the sensitivity of antibiotics on the most numerous species of Staphylococcus

Bacteria isolated from various forms of mastitis (n = 432) and pools milk samples (n = 12), were tested *in vitro* by disc method **(EUCAST, 2014)**, by evaluation of the zones of inhibition to grow on Mueller-Hinton agar after 24 h incubation at 37 °C. To test the sensitivity of staphylococci to fourteen antibiotics; ampicillin 10 μg (28 – 29 mm), amoxicillin 25 μg (28 – 29 mm), cefoperazone 30 μg (14 – 18 mm), cefoxitin 30 μg (23 – 29 mm), cloxacilin 5 μg (10 – 13 mm), erythromycin 10 μg (13 – 23 mm), lincomycin 15 μg (9 – 15 mm), neomycin 10 μg (12 – 17 mm), methicillin 10 μg (9 – 14 mm), novobiocin 5 μg (10 – 13 mm), oxacillin 5 μg (10 – 13 mm), penicillin 10U (28 – 29 mm), streptomycin 10 μg (11 – 15 mm), and tetracycline 10 μg (14 – 19 mm) test discs were used (Oxoid Ltd., Basingstoke, Hants, UK). The choice of antibiotics reflects the range contained in several intramammary products available for treating mastitis in Slovakia. The sensitivity or resistance of the bacteria tested was interpreted according to the reference zones in conformity with the instructions of **EUCAST (2014)**. The tribes *S. aureus* CCM 5973 and *S. epidermidis* 4418 were used as a control in the tests. In view of the abundance of the species of the CPS and CNS, it was only possible for the species *S. aureus* and *S. epidermidis* in practical terms, to evaluate resistance as a percentage: a negligible (<0.1%), very low (0.1 – 1%), low (1 – 10%), moderate (10 – 20%), high (20 – 50%) or very high (50 – 70%).

Statistical analyses

Statistical analysis was performed using the Microsoft Excel 2007 software. Chi-square test (χ^2 test) was used to compare the individual proportions **(Kabrt, 2013)**.

The dependence of the individual signs was tested at a significance level $\alpha = 0.05$, with critical value = 5.991.

RESULTS AND DISCUSSION

Table 1 gives an overview of the bacteria *Staphylococcus* spp., which was isolated from sheep's milk, during the three years (2017 – 2019) on holdings in Slovakia. In the reporting period, 273 coagulase-positive staphylococci were isolated of which 48.7% (133) *S. aureus* was isolated in 8.1% (23) *S. intermedius*, 5.9 % (16) *S. hyicus,* and 37.0% (101) *S. schleiferi.*

S. aureus was isolated during the reporting period, although most frequently at the beginning of the reference period (50). *S. intermedius* was isolated in the two last years, most frequently in the second year (12), S. *hyicus* was isolated in the first (12) and second (4) years of the experiment. *S. schleiferi* was isolated during the reporting period, most frequently at the end of the experiment (48).

In the reporting period, 158 coagulase-negative staphylococci were isolated, of which *S. epidermidis* was isolated in 55.7% (88), *S. chromogenes* 31.0% (49), *S. caprae* 3.8% (6), *S. xylosus* 4.4% (7), other species were rarely isolated.

Table 2 shows the reported overview of the occurrence of resistance to 14 antibiotics in four tested species of staphylococci (n = 371), which were most frequently isolated during the experiment.

Although intramammary infections caused by CoNS are usually self-limiting, there are reports of clinical mastitis cases that often require antimicrobial treatment **(Taponen, 2006; Pieterse and Todorov, 2010)**.

Occurrence to resistance to 14 antibiotics in *S. aureus* and *S. schleiferi*, which were isolated from sheep´s milk during the three-year period is reported in Table 3. In the evaluation, the tests of sensitivity of the two most numerous CPS (*S. aureus, S. schleiferi)* were numerically expressed as numbers of (S) – sensitive, (IM) – intermediate, and (R) – resistant as well as the values of the resistance in percentage (%).

Staphylococcus aureus (Table 2 and Table 3) showed the highest resistance to erythromycin 12.0%, novobiocin 10.5%, and neomycin 9.0%. We may consider that there is an adverse incidence of intermediate susceptibility of penicillin (16), novobiocin, and erythromycin (14 strains), to oxacillin and cloxacillin (12 strains), neomycin (11 strains), and lincomycin (9 strains). For other antibiotics, the incidence of resistant strains of *S. aureus* was relatively low.

From all 101 tested strains of *Staphylococcus schleiferi,* it was discovered that 5.9% resistant was to novobiocin, and 5.0% to erythromycin. Intermediate sensitivity was detected to erythromycin (9), amoxicillin and novobiocin (8), ampicillin, and lincomycin (7), and penicillin, methicilin, and cefoperazone (5 strains). Penicillin antimicrobials were reported to be effective against CoNS infections **(Becker, Heilmann and Peters, 2014; Bhattacharyya et al., 2016)**. However, studies declare an increasing prevalence of antimicrobial resistance in CoNS from clinical mastitis cases **(Schmidt, Kock, and Ehlers, 2015; Beuron et al., 2014)**, including resistance to penicillin, tetracycline, lincomycin, and streptomycin **(Taponen et al., 2006)**.

Recent evidence from Europe indicates insignificant problems of resistance to antibiotics commonly used for cases of mastitis in sheep. **Vautor et al**. **(2009)** reported only sporadic resistance in *S. aureus* isolated in France. **Onni et al**. **(2011)**, in Italy, likewise found limited resistance in *S. epidermidis*, except to penicillin for which the resistance rate was 38%. Similar results were observed in Turkey, where in coagulase-negative isolates from subclinical mastitis only resistance to β-lactams was noteworthy (43%), whilst there was a much smaller frequency of resistance to tetracycline (11%) and even less to other agents **(Ergün et al., 2012)**. Moreover, research in Turkey corroborated these findings, the rate of resistance to penicillin was 27% and to tetracycline 8% **(Unal and Çinar, 2012)**. **Martins et al. (2017)** published similar results; 17% of isolates were resistant to penicillin and 11% to tetracycline. Finally, evidence from Greece was consistent with the abovementioned, as the frequency of resistant isolates was 35% of staphylococcal isolates tested **(Vasileiou et al., 2019)**.

The incidence of the following characters (S, IS, R) was compared in two groups, the most numerous of staphylococci *S. aureus* and *S. schleiferi* using a statistical method, the Chi-squared test. On the significance level $\alpha = 0.05$ (5%) was recorded in fourteen antibiotic substances test value ($G < \chi 2$), the statistical independence of tracked characters was confirmed. The antibiotic substance methicillin, penicillin, and oxacillin when applied, $G > \chi 2$, in the test groups of *S. aureus* and *S. schleiferi,* statistical dependence of the observed characters was confirmed, which means that the occurrence of the characters was not random.

Table 1 Bacteria *Staphylococcus* spp. isolated from sheep milk from two herds of sheep during the three-year period in Slovakia.

Bacteria *Staphylococcus* spp.	**2017**	**2018**	**2019**	**Total**	**%**
S. aureus	43	40	50	133	30.9
S. intermedius	3	12	8	23	5.3
S. hyicus	12	4	-	16	3.7
S. schleiferi	9	44	48	101	23.4
S. caprae	1	4	1	6	1.4
S. epidermidis	47	21	20	88	20.4
S. chromogenes	29	8	12	49	11.4
S. sciuri	3	-	-	3	0.7
S. simulans	3	-	-	3	0.7
S. warneri	-	2	-	2	0.5
S. xylosus	4	-	3	7	1.6
∑	154	135	142	431	100.0

Table 2 Total overview of the incidence of resistance to 14 tested antibiotics in the two species of CPS and CNS staphylococci (n = 371), which were the most frequently isolated from sheep's milk, during the three years.

Bacteria	R	n	Antibiotics													
			AMP	AML	CFP	FOX	OB	E	MY	MET	N	NV	OX	P	S	T
S.aureus (n = 133)	1.	43	4	3	1	1	1	2	3	1	6	8	4	10	1	1
	2.	40	1	2	-	2	1	8	2	-	5	3	1	3	-	-
	3.	50	1	2	1	2	1	6	2	1	1	3	2	3	-	-
	∑	133	6	7	2	5	3	16	7	2	12	14	6	7	1	1
S.schleiferi (n = 101)	1.	9	-	-	1	-	-	-	-	-	-	1	-	-	-	-
	2.	44	-	1	-	-	2	1	-	-	2	1	-	1	11	-
	3.	48	2	1	-	-	-	4	1	-	2	4	-	1	-	-
	∑	101	2	2	1	-	2	5	1	-	4	6	-	2	1	-
Resist. ∑ KPS		234	8	9	3	5	5	21	8	2	16	20	6	9	2	1
S. epidermidis (n = 88)	1.	47	2	2	1	-	1	2	1	-	2	2	-	2	-	-
	2.	21	1	1	1	-	2	1	2	-	2	3	1	2	1	-
	3.	20	-	-	1	-	-	3	1	-	2	2	-	1	-	-
	∑	88	3	3	3	-	3	6	4	-	6	7	1	5	1	-
S. chromogenes (n = 49)	I.	29	-	-	-	-	-	1	1	-	1	1	-	1	-	-
	II.	8	-	-	2	-	-	-	-	-	1	1	-	-	-	-
	III.	12	1	-	-	1	-	-	-	-	-	1	-	1	-	-
	∑	49	3	-	-	1	-	1	1	-	2	3	-	2	-	-
Resist. ∑ KNS (∑n)		137	6	-	3	1	3	7	5	-	8	10	1	75	4	-

Note: (AMP) Ampicillin 10 μg; (AML) Amoxicillin 25 μg; (CFP) Cefoperazone 30 μg; (FOX) Cefoxitin 30 μg; (OB) Cloxacilin 5 μg; (E) Erythromycin 10 μg; (MY) Lincomycin 15 μg; (MET) Methicilin 10 μg; (N) Neomycin 10 μg; (NV) Novobiocine 5 μg; (OX) Oxacillin 5 μg; (P) Penicillin 10 IU; (S) Streptomycin 10 μg; (T) Tetracycline 10 μg.

Table 3 An overview of the sensitivity and the occurrence of resistance to 14 tested antibiotics in *S. aureus* and *S. schleiferi* representatives of the CPS, isolated from sheep's milk in the years 2017 to 2019.

Antibiotics	*S. aureus* (n = 133)				*S. schleiferi* (n = 101)				TEST *
	S	IS	R	%	S	IS	R	%	G
Ampicillin	119	8	6	4.5	92	7	2	2.0	1.165
Amoxicillin	121	5	7	5.3	91	8	2	2.0	3.397
Cefoperazone	126	5	2	1.5	95	5	1	1.0	0.306
Cefoxitin	125	3	5	3.8	97	4	-	-	4.388
Cloxacilin	118	12	3	2.6	95	4	2	2.0	2.357
Erythromycin	103	14	16	12.0	87	9	5	5.0	3.887
Lincomycin	117	9	7	5.3	93	7	1	1.0	3.169
Methicilin	131	-	2	1.5	96	5	-	-	8.153*
Neomycin	110	11	12	9.0	93	4	4	4.0	4.396
Novobiocine	105	14	14	10.5	87	8	6	5.9	2.189
Oxacillin	115	12	6	4.5	97	4	-	-	7.296*
Penicillin	110	16	7	5.3	94	5	2	2.0	7.757*
Streptomycin	127	5	1	0.8	97	3	1	1.0	5.509
Tetracycline	127	5	1	0.8	199	2	-	-	4.512

Note: Sensitivity (S); Intermediate sensitivity (IS); Resistance (R); % resistance on the base n;* χ^2 test (significance level $\alpha = 0.05$ (5%); critical value $\chi 2 = 5.991$; G – testing value).

CONCLUSION

By the bacteriological examination of individual and pool samples of sheep's milk during the three seasons of machine milking was isolated and taxonomically classified 11 species from the total number of 431 bacteria *Staphylococcus* spp. From the coagulation of positive staphylococci (KPS) (n = 273), *S. aureus* was isolated throughout the period considered, but most often in the last year (50). The incidence of *S. intermedius* was highest in the second year (12). *S. hyicus* was isolated in the first and second years of experiment (16) and *S. schleiferi* was most experienced in the last year (48). From the group of coagulase-negative staphylococci (KNS) (n = 158), *S. epidermidis* occurred in 55.7% (88), *S. chromogenes* 31.0% (49), *S. caprae* 3.8% (6), *S. xylosus* 4.4% (7), and other species rarely occurred. *S. aureus* bacteria (n = 133) showed maximum resistance to erythromycin 12.0% and novobiocin 10.5%, and to neomycin 9.0% as well. The incidence of intermediate sensitivity was observed in penicillin (16 strains) of novobiocin, erythromycin (14 strains), oxacillin and cloxacillin (12 strains), neomycin (11 strains), and lincomycin (9 strains). *Staphylococcus schleiferi* bacteria (n = 101) showed the highest resistance to novobiocin (5.9%) and erythromycin (5.0%), however, a high incidence of intermediate susceptibility to erythromycin (9), amoxicillin and novobiocin (8), ampicillin and lincomycin (7), penicillin, methicilin and cefoperazone (5 strains) can be identified as adverse. This work was aimed at testing the most frequent representatives of the genus *Staphylococcus* spp., a relatively adverse development of resistance to the most commonly used antibiotics for the treatment of inflammation of the udder in sheep.

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MILK THISTLE FLOUR EFFECT ON DOUGH RHEOLOGICAL PROPERTIES

Tatiana Bojňanská, Alena Vollmannová, Janette Musilová

ABSTRACT
The influence of the addition of partially defatted milk thistle seed flour was studied by analyzing the rheological properties of dough in order to further exploit the functionality of partially defatted milk thistle flour in bakery products. The rheological properties of dough were monitored using Mixolab 2 (Chopin Technologies, France). A rheofermentometer F4 (Chopin Technologies, France) was used to check the dough fermentation, and for the baking trials wheat flour, rye flour, and milk thistle flour were kept in the portion: 50:50:0 (control flour); 50:45:5; 50:40:10 and 50:35:15. The addition of different milk thistle flour in the mixtures resulted in a difference in the viscoelastic properties of the dough. The results showed a weakening of the gluten network in all trial mixtures evaluated. The dough development time values of the control flour were 1.20 min, while an addition of milk thistle flour in portions of 5, 10, and 15% increased these values to 1.30 min, 1.90 min, and 2,80 min, respectively. In addition to higher dough development time values, all trial mixtures exhibited also higher stability (5.07 min; 6.25 min and 8.03 min), when compared to the control flour (4.63 min). The trial mixture with 15% milk thistle flour had different characteristics of gelatinization and retrogradation. The rheofermentometer measured the dough characteristics during proofing, and the trial mixtures with the addition of MTF had a retention volume at approximately the same level as the control flour (WRF). The Volscan profiler was used to determine the bread volume and other parameters. All breads had high volume and specific volume values and can be rated as good, with good porosity and ratio. Mixtures containing 5%, 10% and 15% milk thistle flour added to wheat flour + rye flour maintained rheological parameters within the recommended limits for good technological behavior and, consequently, good quality of bakery products. From all of the above data, it can be stated that, with regard to their baking characteristics, these flour mixtures fall into the category of flours suitable for bakery products.

Keywords: wheat-rye-milk thistle composite; Chopin+ protocol; rheofermentometer; bread quality

INTRODUCTION
Food not only supplies human beings with nutritive components that are indispensable for maintaining a healthy daily life, it also contains many types of biofunctional components that help to maintain good physical and mental health. In the last three decades, a growing interest has occurred in the use of active compounds from natural sources that can contribute to promoting consumer's health and preventing diseases. One opportunity is milk thistle (*Silybum marianum* L. Gaertn); an annual or biennial plant belonging to the family *Asteraceae* and usually growing in dry, sunny areas. The plant native to the Mediterranean area has now spread to other warm and dry regions. It is a plant that has been used in folk medicine for over 2,000 years as a remedy for a variety of medical conditions, especially the liver, kidney, and gallbladder ailments **(Anthony et al., 2013; Eskandari Nasrabadi et al., 2014; Wang et al., 2014; El-haak, Atta and Abd Rabo, 2015; Saad-Allah, Fetouh and Elhaak, 2017; Abenavoli et al., 2018)**.

In Europe, it is cultivated widely for the production of raw materials used in the pharmaceutical industry, especially in Austria, Germany, Hungary, and Poland. Over-the-counter nutritional or dietary supplements are becoming extremely popular in the United States, Europe, and many other countries for liver enhancement and recovery **(Anthony et al., 2013; Jedlinszki et al., 2016; Salomone, Godos and Zelber-Sagi, 2016; Choe et al., 2019; Çeribasi et al., 2020)**.

Milk thistle is a rich source of ingredients, such as amino acids, fatty acids, minerals, and phytochemicals exhibiting nutraceutical effects on human health. The whole plant is used for medicinal purposes. Its seeds (there is confusion about whether milk thistle has fruits or seeds – botanically correct, this plant has a cypselae, which looks like a seed but is technically a fruit.) are rich in proteins, lipids, and total carbohydrates, with concentrations from 19.1 to 30.0%, 20.0 to 30.0%, and 24.2 to 26.3%, respectively. Furthermore, the seed proteins have markedly high amounts of essential amino acids such as lysine, isoleucine, leucine, valine, and threonine, however, they are a poor source of prolin and histidin **(El-haak, Atta and Abd Rabo, 2015; Apostol et al., 2017; Saad-Allah, Fetouh and Elhaak, 2017)**.

In milk thistle seeds linoleic and oleic acids are the predominant fatty acids in its oil **(Tuğba- Çelik and Gürü, 2015)** (Table 1). Milk thistle oil has significant scavenging effects on the DPPH radical. This antioxidant activity reflects the bioactive molecules composition in oil extracted from milk thistle (1.1 ±0.6% of polyphenols on dried material, 13.4 $mg.100g^{-1}$ beta-carotene of carotenoids and 70 – 85% of unsaturated fatty acids) **(Li et al., 2012; Anthony et al., 2013; Rahal et al., 2015; Abenavoli et al., 2018)**.

Silymarin represents 1.5 – 6% of the fruit's dry weight and is an isomeric mixture of unique flavonoid complexes – flavonolignans **(Eskandari Nasrabadi et al., 2014; Bijak, 2017)**. The main representatives of this group presented in silymarin are silybin, isosilybin, silychristin, isosilychristin, silydianin, and silimonin. The chemical composition of milk thistle fruit besides flavonolignans also includes other flavonoids, such as taxifolin, quercetin, dihydrokaempferol, kaempferol, apigenin, naringin, eriodyctiol, and chrysoeriol **(Kvasnička et al., 2003; Bijak, 2017)**. The whole plant is used for medicinal purposes, but the highest content of silymarin is in the seeds. Silybin, a secondary metabolite isolated from the seeds of milk thistle was discovered as the first member of a new family of natural compounds called flavonolignans in 1959 **(Biedermann et al., 2014)**. Since the 1970s, silybin has been regarded in official medicine as a substance with hepatoprotective properties. There is a large body of research that demonstrates silybin's many other healthy properties, but there is still a lack of papers focusing on its molecular structure, chemistry, metabolism, and novel form of administration **(Bijak, 2017)**.

Currently, the most important medicinal application of milk thistle is its use as a hepatoprotective, antioxidant, antiradical, and free radical scavenging food supplement. Silymarin is a natural antioxidant and this action is believed to contribute to the hepatoprotective effects of milk thistle preparations. Cold-pressed milk thistle seed flour was evaluated for its phytochemical composition, and gut microbiota modulating, free radical scavenging, anti-inflammatory, and anti-proliferative capacities **(Surai, 2015)**. The results suggest milk thistle seed flour's potential health benefits in functional foods **(Choe et al., 2019; Menasra and Fahloul, 2019)**, despite its properties observed in experimental studies, the current efficacy of milk thistle preparations in patients with liver diseases is not fully compelling **(Salomone, Godos and Zelber-Sagi, 2016; Abenavoli et al., 2018)**.

Cytotoxic activities of silychristin, silydianin, and silybinin were evaluated against Caco-2 cells (colon cancer cell line) **(Rahal et al., 2015)**, but the anticancer activity of silymarin, as well as silibinin, was demonstrated against various cancer cells such as breast, skin, cervix, ovary, prostate, lung and hepatocellular cancers **(Bosch-Barrera and Menendez, 2015)**.

Most of the existing researches on the milk thistle plant dealt only with pharmacological and medicinal studies due to the production of silymarin and its use for healing some hepatic diseases. However, the defatted milk thistle seed flour which is a by-product, and is typically eliminated as waste, may contain beneficial components such as proteins, carbohydrates (especially crude fibres), minerals, and some phytochemicals and be utilized as a suitable food ingredient in low fibre food **(Li et al. 2012; El-haak, Atta and Abd Rabo, 2015)**. Milk thistle leaves and flowers have been used as a vegetable for salads and a substitute for spinach. On the other hand, milk thistle seeds are roasted for use as a coffee substitute.

The fundamental food in many parts of the world is bread. Wheat flour dough is a viscoelastic system that exhibits an intermediate rheological behavior between a viscous liquid and elastic solid. The viscoelastic protein network plays a predominant role in dough processing as well as in textural characteristics of the finished bread. The gluten network in wheat is constructed from gliadins and glutenins, which are responsible for the dough resistance to extension, thus providing the dough with its unique viscoelastic properties and its ability to retain gases produced during the yeast fermentation process. Therefore, gluten is a fundamental component, which is responsible for the overall quality and structure of bread **(Fendri et al., 2016)**.

The information necessary for the evaluation of the flour quality is obtained on the basis of some indices determined through the chemical, rheological and technological analyses. Rheological characteristics, such as elasticity, viscosity, and extensibility, are important for the milling and bakery industry, used for a prediction of the dough processing parameters and the end product quality **(Jirsa, Hrušková and Švec, 2007; Ziobro et al., 2013; Švec and Hrušková, 2015)**.

Composite flours on the basis of wheat and other cereals and non-grain seeds became popular in the baking technology. The purpose of this research was to identify the percentage of bread flour as well as to find a mixture of flours, the composition of which combines the nutritional value with adequate processing properties in an optimal way. The objective of this work was to characterize mixtures of wheat flour + rye flour, with a replacement of 5, 10, and 15% of milk thistle seed flour, with regard to the technological characteristics of dough (rheological properties) and objective properties of the final product.

Scientific hypothesis

We expect some effect of milk thistle seed flour in addition to baker's flour (wheat flour and rye flour) on the rheological properties of the dough, its ability to retain fermentation gases, and the objective properties of the final product.

MATERIAL AND METHODOLOGY

In this study, commercial blend flours (Miroslav Grznár MLYN ZRNO, Slovak Republic), were used: defatted milk thistle seed flour (8.4% fat) in mixtures with wheat flour (ash 0.65%) and rye flour (ash 0.96%). Three types of mixtures of wheat flour + rye flour and different proportions of partially defatted milk thistle seed flour were obtained, and a control flour without milk thistle flour in the following ratios: 50:45:5, 50:40:10, 50:35:15, and 50:50:0, respectively. Further materials for dough and bread formulation were: salt (K.S. Czech Republic, a. s.), sucrose (Slovenské cukrovary s. r. o.), and dry yeast of the species *Saccharomyces cerevisiae* (Ruf, sušené droždie).

Rheological properties of dough were monitored using Mixolab 2 (Chopin Technologies, France) applying the "Chopin+" protocol. The international standard ICC-Standard Method No. 173, a protocol for complete characterization of flours, was used, and a simplified graphic interpretation of the results was performed. In „Chopin+" protocol Mixolab recorded changes of torque in five defined points as follows: C1 – water absorption; C2 – weakening of the protein-based on mechanical stress at increasing temperature; C3 – the rate of starch gelatinization; C4 – stability of the formed gel; C5 – starch retrogradation during the cooling period.

A Rheofermentometer F4 (Chopin Technologies, France) was used to check the dough fermentation, to measure the dough parameters: the maximum dough height (Hm, mm), the maximum height of the gas release curve (Hm′, mm), the time required to obtain H´m (Tx, hours), total volume (total volume of gas produced in mL), the volume of CO_2 lost (carbon dioxide volume in mL that the dough has lost during proofing), retention volume (carbon dioxide volume in mL still retained in the dough at the end of the test). The dough (315 g) was placed in a movable basket of the gas meter with a 2000 g cylindrical weight, and the cover of the vat was fitted with an optical sensor. The test was conducted at 28.5 °C for 3 h (according to the conditions of the Chopin protocol reported in the rheofermentometer instruction manual). The method conforms to the AACC 89-01 **(AACC, 2000)** standard for the measurement of yeast activity and gas production.

Bread making procedure: for baking trials wheat flour, rye flour, and milk thistle flour (MTF) were kept in the portion: 50:50:0 (control flour WRF); 50:45:5; 50:40:10 and 50:35:15. Other recipe compounds were: water (the level of water used in baking experiments, to obtain the dough suitable for bread baking – the water addition to individual dough mixes ranged between 61 to 64%, 2.0% NaCl, and 1.4% dry yeast. The percentages are based on 100% of the flour mixture. All ingredients were kneaded 3 minutes at lower speed and 4 minutes at higher speed in a spiral kneader type SP 12 D (Diosna Dierks & Söhne GmbH, Osnabrück, Germany). After 40 min fermentation (35 °C) samples were baked for 40 min in mode: 180 °C 7 minutes, 200 °C 20 minutes, and 160 °C 13 minutes, with stream (250 mL) (MIWE Condo). The bread loaves were cooled at room temperature and analyzed by a Volscan Profiler volume analyzer (Stable Mycrosystems, Surrey, UK) one hour after baking (weight of the bread (g), bread volume (mL), specific volume ($mL.g^{-1}$), volume-yield ($mL.100g^{-1}$ flour), the aspect ratio of a middle slice ect.).

Statistical analysis

The results of the technological measurement were statistically analyzed using XLSTAT for Excel (version 2015). The data were subjected to the Z. test at significance level 0.05 to determine differences between samples.

RESULTS AND DISCUSSION

The effect of adding milk thistle flour on the dough properties and bread parameters was investigated.

Bread is widely consumed as a staple food across many cultures and countries worldwide. There are three major stages of bread making: mixing, fermentation (i.e. proofing), and thermal setting (i.e. baking/steaming). The mechanical energy imparted during mixing induces the formation of a viscoelastic dough matrix. Besides the proper development of the gluten network, the initial gas inclusion during mixing and the yeast activity during proofing also greatly affect the bread quality **(Gao et al., 2017)**. Gluten is a major protein component of the same cereals which is responsible for flour processing characteristics in the bakery industry. The reduction of gluten often results in baked bread with a crumbling texture, poor color, and other post-baking quality defects **(Schmiele et al., 2017)**.

In the trial breads prepared with milk thistle, the proportion of gluten gradually decreased with an increasing amount of the addition, which affected the rheological behavior of the dough. The preliminary rheological analysis indicates the influence of applied MTF on the dough properties.

Monitoring the rheological properties of dough is very important for the overall technology to estimate the mechanical properties of dough and to imitate its behavior during its processing or even to anticipate the quality of the final product **(Dapčević-Hadnađev et al., 2014; Torbica, Belović, and Tomić, 2019)**.

The Mixolab measures in real time the torque (expressed in Nm) produced by the passage of the dough between the two kneading arms, thus allowing the study of rheological and enzymatic parameters: dough rheological characteristics (development time, hydration capacity, etc.), protein reduction, enzymatic activity, gelatinization and gelling of starch. Figure 1 exhibits the Mixolab curve (Chopin+ protocol) and parameters of the analyzed sample (WRF 50:50 = control flour). The results confirm that the control flour has a short dough development time (1.2 min) and only average stability (4.63 min), which is due to its composition, especially the high proportion of rye flour which cannot form gluten. During the heating and cooling stages, the control flour showed a high viscosity of starch gelatinization and retrogradation.

The addition of different MTF (5%, 10%, 15%) in the mixtures resulted in a difference in the viscoelastic properties of dough (Figure 2). The results showed a weakening of the gluten network in all trial mixtures evaluated.

Water absorption, amplitude, dough development time, stability, and C1 Mixolab parameters are used to evaluate the gluten network formation, dough development during mixing, and stability at constant mechanical shear. The first stage of the Mixolab curve is related to the farinograph analysis, which allows evaluating the viscoelastic properties of dough including hydration and mixing tolerance index. In Mixolab, the first stage temperature is kept at 30 °C. The mixing process after the first stage may give extra energy to the dough system; therefore, these results should be used with caution because they do not represent the breadmaking process, since the dough can no longer pass through the mixing process after the formation of the gluten network, without weakening the structure during the mixing of the dough **(Schmiele et al., 2017)**.

Table 1 Fatty acid composition milk thistle oil, %.

	Fatty acid	%		Fatty acid	%
C 18:2	**Linoleic acid**	**54.97**	C 20:1	Gadoleic acid	0.95
C 18:1	**Oleic acid**	**24.10**	C 24:0	Lignoceric acid	0.59
C 16:0	Palmitic acid	8.15	C 18:3	Linolenic acid	0.17
C 18:0	Stearic acid	5.51	C 16:0	Palmitoleic acid	0.10
C 20:0	Arachidic acid	3.03	C 14:0	Myristic acid	0.09
C 22:0	Behenic acid	2.27	C 17:0	Margaric acid	0.07
			C 17:1	Heptadesenoic acid	0.03

Note: **(Tugba-Celik and Gürü, 2015)**.

Table 2 Rheofermentometer parameters of dough from the trial mixtures and the control flour.

Sample	H´m (mm)	Tx (time)	Total volume (mL)	Retention volume (mL)	Volume of CO^2 lost (mL)	Retention coefficient
Control flour (50:50:0)	60.50a	0:54:00a	1 569a	1 247a	322a	79.50a
50:45:5	74.20b	0:34:30b	1 779b	1 230a	549b	69.10a
50:40:10	79.30b	0:39:00a	1 803b	1 228a	575b	68.10a
50:35:15	83.50b	0:46:30a	1 927b	1 307a	621b	67.80a

Note: The following indices were measured: H´m – maximum height of the gas release curve; Tx – time required to obtain H´m; Total volume – volume of gas produced, Retention volume – CO_2 volume in mL still retained in the dough at the end of the test; Volume of CO_2 lost – CO_2volume in mL that the dough has lost during proofing; Gas retention coefficient (Retention volume/Total volume). Means followed by different letters are significantly different *(p* <0.05).

Table 3 Bread quality parameters.

Trial	Weight, g	Volume, mL	Specific volume, $mL.g^{-1}$	Volume-yield, $mL.100g^{-1}$ flour	Aspect ratio of middle slice
Control bread (WRF)	805a	2 126a	2.64a	425.17a	0.68a
Bread with 5% MTF	964b	2 040a	2.12a	408.02a	0.75a
Bread with 10% MTF	883b	1 749b	1.98a	349.88b	0.64a
Bread with 15% MTF	881b	1 734b	1.97a	346.80b	0.87a

Note: Means followed by different letters are significantly different (*p* <0.05).

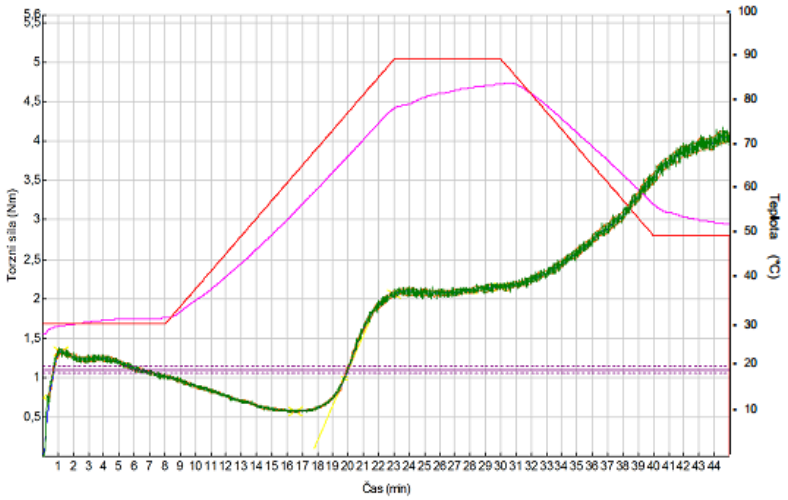


Figure 1 Mixolab curve Chopin+ protocol, control flour (WRF 50:50).

The rheological properties are provided in Figure 3. The dough development time values of the control flour were 1.20 min, while addition milk thistle flour increased these values to 1.30 min, 1.90 min, and 2.80 min, respectively. In addition to higher dough development time values, all trial mixtures exhibited also higher stability (5.07min; 6.25 min and 8.03 min) when compared to the control flour (4.63 min). In general, the increase of the dough development time indicates that higher fibre content slows down the rate of hydration and the development of gluten **(Kohajdova et al., 2011)**. At large, the stability of the dough is attributed to protein poor in sulfhydryl groups, which normally causes dough softening or even degradation. The gluten network is stronger and has higher resistance to shear stress at higher protein content **(Kaur et al., 2016)**. Although milk thistle flour contains a high level of protein, a higher level of fibre may weaken the gluten network **(Shahat Mohamed, Hassein Ahmed and Hady Essam, 2016)**.

The dough development time parameter and stability are very important for bread making because the development of the gluten network should be optimized to ensure loaves with a high volume and soft texture **(Rosell, Collar, and Haros, 2007)**. The enhancing ability of MTF is surprising as this raw material does not bring into the system gluten-forming proteins. Nevertheless, for flours with high rye content, the MTF had, besides its nutritional benefits, also a technological benefit related to a higher mixing tolerance (Figure 2). Similar results were found by other authors **(Apostol et al., 2017; Bortlíková, Kolarič, and Šimko, 2019)**.

The parameters slope-α, C2, and C2-C1 were recorded when the heating reached 52 – 57 °C at the beginning of the second stage. In this phase, starch started to gelatinize, and the proteins changed their quaternary, tertiary, and secondary structures due to protein denaturation. Also, swelling of the starch granules was observed, mainly for the damaged granules, and the changes along the mixing process modified the dough consistency **(Schmiele et al., 2017)**. C2 values, representing the weakening of the protein network, decreased from 1.332 Nm to 0.571 Nm (control flour), or from 1.572 Nm to 0.774 Nm (50:35:15), and then remained at an almost constant value indicating a certain compatibility level of gluten and milk thistle flour proteins during the dough formation.

In the third stage, where the evaluated parameters were slope-β, C3, and C3-C2, the heating step began until reaching 70 °C while the dough remained under constant mixing, stopping protein denaturation and starch gelatinization. Higher C3 values were observed for all trial mixtures when compared to the control flour, but the difference for consecutive samples is rather small.

The fourth stage assessed the parameters slope-γ, C4, and C4-C3, and evaluated the enzymatic activity and heat stability of the starch gel, at temperatures above 80 °C. In this sense, a dependence of the determined values on the dough formulation was sought. A decrease in C4 was observed in the trial mixture 50:35:15 (15% milk thistle flour), and the torque value was significantly lower compared to all other samples during the last stage too.

In the last stage the dough was cooled to 58 – 60 °C, and the parameters C5 and C5-C4 were assessed. The retrogradation stage of starch (C5) for the tested WRF and MTF mixtures demonstrated similar differences as for the starch gel stability. Significant differences in C5 can be seen between the trial mixture 50:35:15 and all other samples.

The trial mixture 50:40:10 (10% MTF) presented the highest C5 values, exhibited the highest retrogradation in the cooling phase, due to the higher degree of starch gelatinization in the heating phase. The differences between this trial mixture, the mixture 50:40:5, and the control flour 50:50:0 were small. This is also documented in Figure 4, because no changes in slope-α and slope γ was observed for the trial mixture 5%, 10%, and 10% MTF, and the control flour. The results from slope-β confirmed that the trial mixture 50:35:15 (15% MTF) has different gelatinization characteristics at a temperature above 52 – 57 °C.

The rheofermentometer analysis of flour and dough enables accurate simulation of the processing conditions during the production of baked goods containing yeast **(Dapčevič-Hadnađev et al., 2011)**. The rheofermentometer measures the characteristics of dough during proofing (the dough development, the production of gas due to yeast action, the porosity of the dough, the tolerance of the dough during proofing), and the analysis determines the total gas production of yeast and dough volume at standard barometric pressure over time. The instrument records two curves during the dough fermentation and rising, one describing the development of the dough and another depicting the production and retention of gas.

Dough development is a function of both yeast gassing power and gluten network integrity. The rheofermentometer analysis allows simultaneous observation of both yeast fermentation and dough growth, which provides direct evidence on the correlation between the two factors. The dough development curves of the control sample (WRF) and trial mixture sample with the addition of MTF 15 % are shown in Figure 5 and Figure 6, respectively. It is evident that the trial mixture dough sample with the addition of milk thistle 15% rose to a greater height (83.5 mm) than the control dough (60.5 mm).

The rising of the control dough can be characterized as insufficient and slow, which is associated with a high proportion of rye in the flour. The results recorded throughout the tests are shown in Table 2, and it can be concluded that the addition of MTF to bread flour (wheat flour and rye flour) influenced all evaluated parameters.

Rheofermentometer curve trial mixtures with MTF 15% showed that the dough after the fermentation completely retained 67.8% of the total CO_2 produced. In comparison, the rheofermentometer curve WRF retained 79.5% of the total CO_2 produced. The maximum dough volume MTF 15% of 1927 mL was reached after 1 hour, 10 minutes, and 30 seconds, in comparison with WRF (1569 mL after 1 hour and 15 minutes).

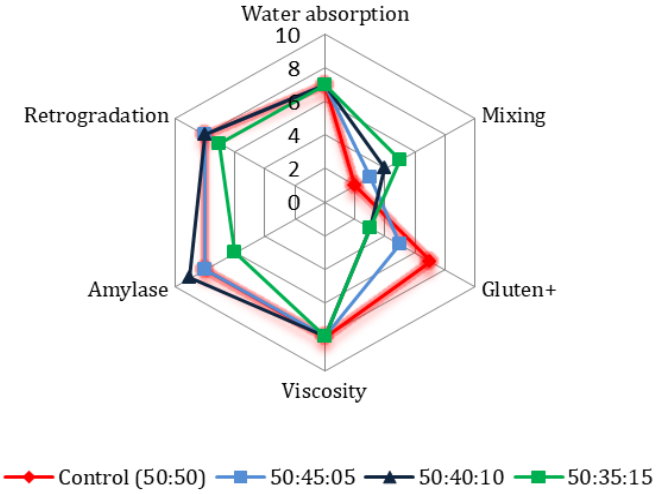
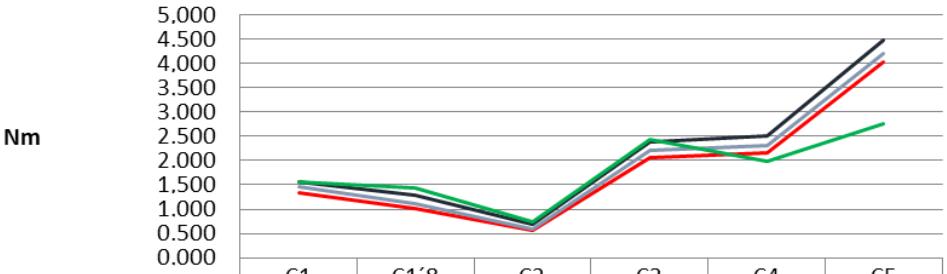


Figure 2 Mixolab profiler.



	C1	C1´8	C2	C3	C4	C5
Control (50:50)	1,332	1,015	0,571	2.061	2,149	4,034
50:45:05	1.460	1,109	0,593	2,214	2,303	4,201
50:40:10	1.564	1.293	0.690	2,393	2,501	4,477
50:35:15	1,572	1.445	0,744	2.423	1,981	2,749

Figure 3 Influence of milk thistle seed flour added to wheat flour + rye flour in different proportions in Mixolab characteristics (torque during mixing).

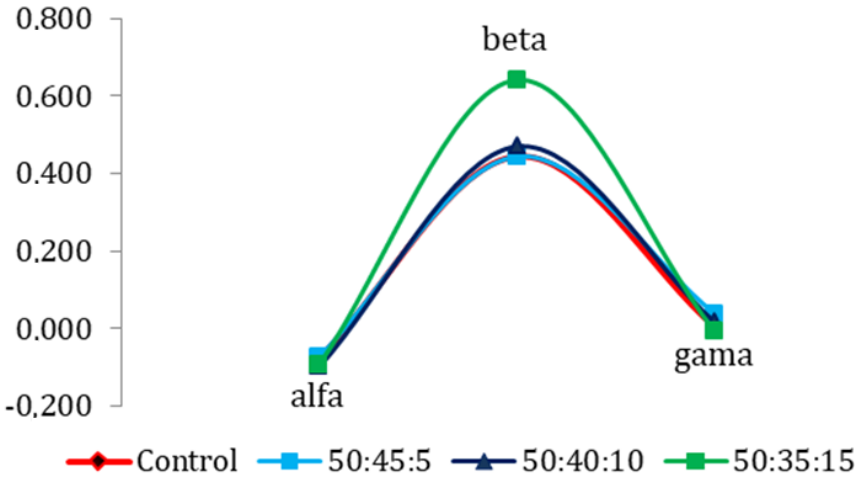


Figure 4 Slope alfa, beta and gamma during the mixing process of dough.

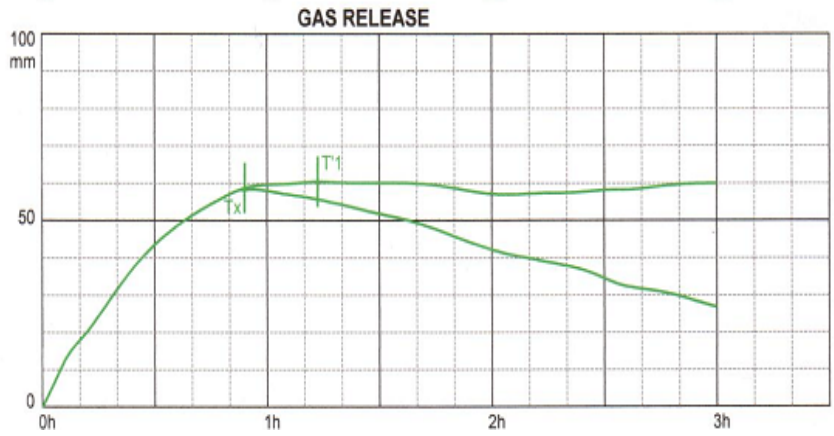


Figure 5 Rheofermentometer curve WRF.

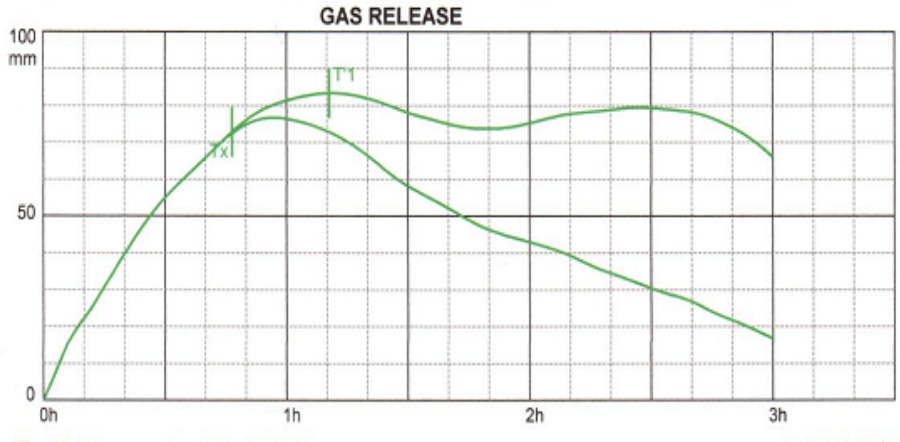


Figure 6 Rheofermentometer curve trial mixtures with MTF 15%.

a **b** **c** **d**

Figure 7 Bread volume and bread aspect ratio by Volscan profiler.
Note: a – control bread; b – bread with 5% MTF; 7c – bread with 10% MTF; d – bread with 15% MTF.

In general, the trials with the addition of MTF began to release CO_2 too early, probably related to the reduction of gluten, but the total gas produced in these samples was higher than in the control flour (Table 2). During rheofermentometer measurements, the trial mixtures with the addition of MTF have lost significantly higher amounts of CO_2 in comparison with the control flour, which is documented by the retention coefficient lower by 13.1%, 14.3%, and 14.7%, respectively. However, it is important to note that the retention volume at the end of the measurement was at approximately the same level as the control flour (WRF) in all evaluated mixtures, with an MTF addition of 15% even higher than the control flour.

Monitoring the rheological properties of dough is very important for the overall technology to estimate the mechanical properties of dough and to imitate its behavior during its processing or even to anticipate the quality of the final product **(Dapčevič-Hadnađev et al., 2011)**. From all of the above data, it can be stated that, with regard to their baking characteristics, these flour mixtures fall into the category of flours suitable for bakery products.

Bread volume is one of the major quality-determining criteria for bread quality. Bread with a larger volume is

always preferred. A Volscan profiler was used to determine the bread volume, and the result was divided by the sample's weight to obtain its specific volume. The form ratio was also calculated by taking the maximum height divided by the maximum width of the sample, of which the measurements were determined by the Volscan profiler.

As can be seen in Figure 7 (by Volscan) and Table 3, bread volume and specific volume decreased with increased addition of MTF. This could be explained by a better gas retention ability of the gluten matrix in the dough, leading to bread with a larger volume in the control sample. The specific volume of bread decreased when the MTF was added. With 2.12 $mL.g^{-1}$, the specific volume of bread with 5% MTF approaches the value of standard wheat-rye bread (2.64 $mL.g^{-1}$) the most. In comparison, commercial wheat and bread with lower gluten content can only achieve a higher porosity by adding various supplements, which has a negative impact on nutrition and consumer acceptance. According to **Gao et al. (2017)**, the structural and textural attributes of baked bread were significantly correlated with the gluten development and the gas production of the dough. These findings suggest a great dependence on baked bread quality on its gluten network. **Kondakci, Wenjuan-Zhang, and Zhou (2015)** state that the low-, medium- and high-protein bread samples showed lower specific volumes compared to their corresponding control sample.

In general, all breads had high specific volume values and can be evaluated as of good quality, with good porosity and ratio. Even the nutritionally interesting addition of 15% MTF did not cause an important decrease in the quality of the trial bread, giving a good presumption for the application of MTF in baking flour.

The addition of MTF affects the color of the flour as indicated by **Apostol et al. (2017)**, color differences between the control flour and the flour mixtures are noticeable with the human eye. The flour mixtures with the highest percentage of added ingredient showed significantly higher redness values compared to the control flour. As the percentage of added partially defatted milk thistle seed flour increased, the color of the flour mixtures darkened compared to pure wheat flour. The overall acceptability of all experimental breads was evaluated as good.

From a nutritional point of view, in addition to silimarin MTF is a valuable source of minerals, especially calcium, magnesium, iron, and potassium. The mineral contents of the defatted milk thistle seed compared to wheat flour were referred to by **Apostol et al. (2017)**. The authors confirmed that compared to the low mineral content of wheat flour, samples of milk thistle seed flour were having higher levels of minerals. MTF is a good source of proteins, mineral compounds, and fats and therefore it can be used for human nutrition. Moreover, due to the high content of flavonolignans, summarily called silymarin, and their positive effects on the liver, it is also suitable for the production of functional foods **(Bortlíková, Kolarič, and Šimko, 2019; Menasra and Fahloul, 2019)**.

The addition of MTF to bread is in line with the trend of designing food according to consumers' needs, also those with special needs. Food thus becomes an active part of a progressive lifestyle and can help prevent certain diseases.

CONCLUSION

Modern foods are expected to contain nutritive components that are indispensable for maintaining a healthy daily life, but also contain many types of biofunctional components that help to maintain good physical and mental health. One opportunity is milk thistle (*Silybum marianum* L. Gaertn). It is a plant that has been used as a remedy for a variety of medical conditions, especially liver, kidney, and gallbladder ailments. The defatted milk thistle seed flour could be utilized as a suitable ingredient in food production, for example in the composite flours based on wheat and other cereals and non-grain seeds that have become popular in the baking technology.

The analysis of the viscoelastic properties gives information about the influence of the applied ingredients on the rheological behavior of the dough. Preliminary rheological analyses indicated a strong influence of the applied non-wheat flour on the dough properties.

Summarising the results and findings of this study, the following conclusions can be postulated:

-The suitability of WRF replacement with MTF in the range 5, 10, and 15% were studied for the preparation of bread as healthy bakery products,

-During the experiments, the effects of the WRF replacement on the technological properties of the dough were determined using Mixolab characteristics. The results showed that the trial mixture with 5 and 10% MTF had very similar results to the control flour (WRF). The trial mixture with 15% MTF has different characteristics of gelatinization and retrogradation,

-A rheofermentometer measures the dough characteristics during proofing, and the trial mixtures with the addition of MTF have created a large gas volume. During the rheofermentometer measurements, the MTFs lost significantly higher amounts of CO_2 compared to the control flour, but the retention volume at the end of the measurement was approximately the same as with the control flour (WRF) at all evaluated mixtures,

-The rheological analysis to predict the quality of the final product showed that it was possible to make bread with a WRF replacement by these functional ingredients in concentrations that allow maintaining the specific volume similar to the control bread,

-A Volscan profiler was used to determine the bread volume and other parameters and confirmed the predicted properties because all breads had high volume and specific volume values and can be evaluated as of good quality, with good porosity and ratio.

The main conclusion in our study concerning the rheological properties of dough WRF and mixtures of WRF with MTF is that the rheological parameters were maintained within limits that can assure a good technological behavior towards obtaining high-quality bakery products.

From all of the above data, it can be stated that, concerning their baking characteristics, these flour mixtures fall into the category of flours suitable for bakery products. The results suggest that milk thistle seed flours have health benefits potential to be used in functional foods.

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THE CONSUMER BEHAVIOR OF SLOVAK MILLENNIALS IN THE SEGMENT OF MILK AND DAIRY PRODUCTS WITHIN PRIVATE LABELS

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ABSTRACT
Despite the fact that the dairy industry has an important position in the food industry (with an 18% share in its total production and sales), dairy businesses are currently struggling with low consumption of milk and dairy products. According to most researchers, the low consumption of milk and dairy products is mainly due to poor eating habits and, last but not least, insufficient promotion of these products. There is also an opportunity for private labels, which are recently on the rise, for which milk and dairy products are the most commonly purchased category of food. This paper aimed to find out how Slovak millennials perceive private labels, in which categories they buy them, what motivates them and on the contrary, discourages them from the purchase, etc. As the main research method, there was chosen the method of anonymous questionnaire survey involving 549 respondents from all over Slovakia; which was subsequently supplemented with a blind test. The submitted results of research declared that our goals were fulfilled and the following conclusions can be stated: Slovak millennials buy private labels in particular in the category of milk and dairy products, perceive private labels as an adequate alternative to their purchase and when purchasing milk and dairy products, they are mostly influenced by recommendations of family and friends and the tasting.

Keywords: private labels; millennials; milk and milk products; blind test

INTRODUCTION
The dairy industry has an important position in the food industry of the Slovak Republic, as it accounts for 18% of its total production and sales. Like production, sales are mostly influenced by the evolution of raw cow's milk prices on both, European and world markets. Prices had a significant impact on the industry in 2009 – 2010, as there were observed sharp fluctuations – while 2009 brought to the dairy sector a profit of 2.8 million €; in 2010, it showed a loss of 7.3 mil. €. The following years did not bring any change and it can be said that the sector is loss-making **(MPRV SR, 2018)**.

The average purchase price of raw cow's milk was the lowest in 2009 when it was around 20.82 €/100 kg. The highest price was reached in 2014, at 33.82 €/100 kg. While the development of average purchase prices of raw cow's milk excluding VAT is in the graphical form shown in Figure 1, Figure 2 shows the production of milk and dairy products in the Slovak Republic.

The raw cow's milk production showed a fluctuating trend over the considered period between 2009 and 2019. The highest production was achieved in 2012 at the level of 959.4 thous. t. On the contrary, the lowest milk production was in 2010, namely 918 thous. t.

Despite the fact that milk and dairy products take an irreplaceable place in everyday food consumption, especially because the milk is a complex food, what is confirmed by its nutritional content, vitamins, magnesium, and iron, which are indispensable in the daily diet **(Nicklas et al., 2009)**. Some ingredients are included in milk in a higher amount, others in a more convenient form to be used by the organism.

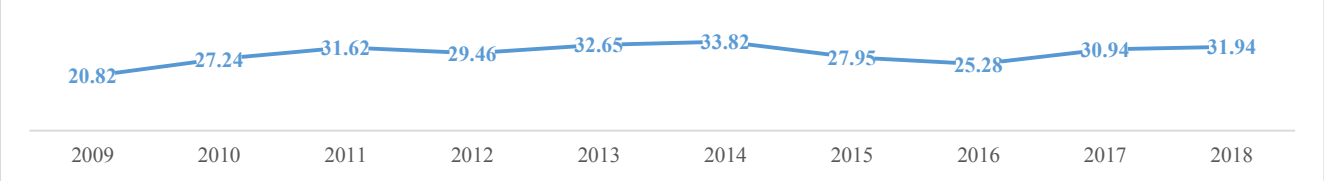


Figure 1 Development of average purchase prices of raw cow 's milk excluding VAT (€/100 kg). Note: Source – **VÚEPP (2019)**.

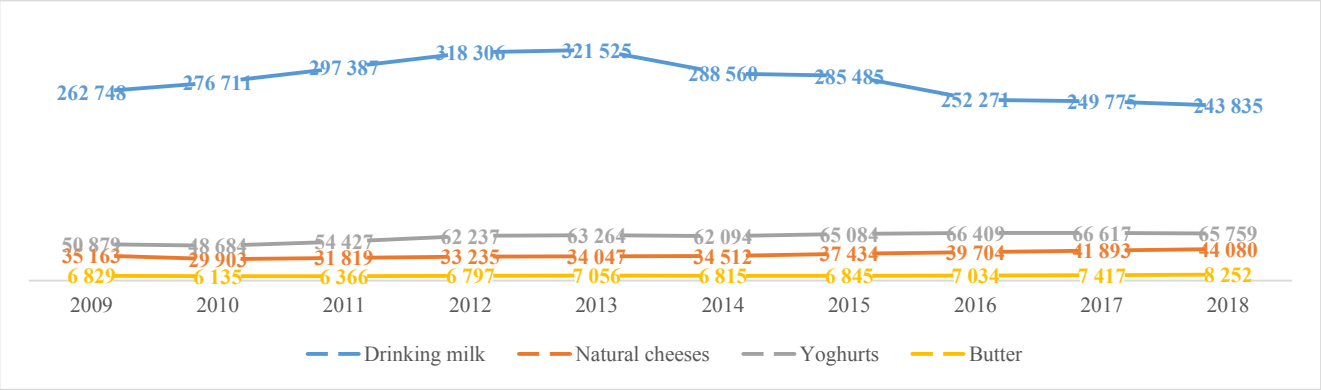


Figure 2 Production of milk and dairy products in Slovakia (t). Note: Source – **VÚEPP (2019)**.

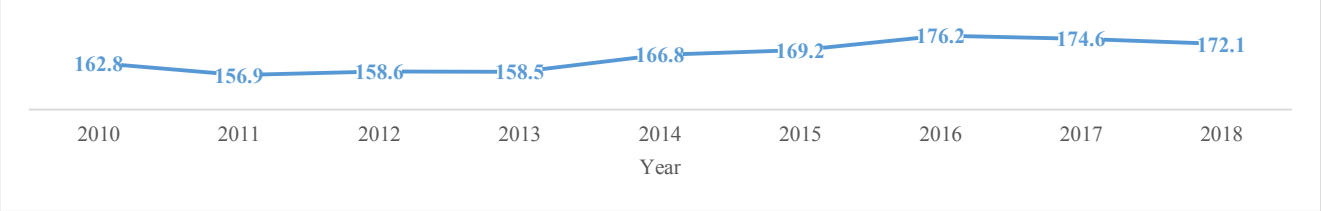


Figure 3 Average milk and dairy consumption per capita (kg/year). Note: Source – **VÚEPP (2019)**.

At the forefront of this, it is calcium that is bound in milk in organic compounds and therefore can be easily used by the growing organism **(The National Dairy Council, 2008)**, nowadays dairy companies are struggling with low milk and dairy consumption – as can be seen from the Figure 3, the consumption of milk and dairy products per capita peaked at 176.2 kg in 2016 and have been rather negative since then.

According to the results of several researches (e.g. **Kubicová and Habánová, 2012; Krivošíková et al., 2019; Kubicová et al., 2019,** etc.), the low consumption of milk and dairy products is caused mainly by poor eating habits of consumers and, last but not least, insufficient promotion of these products. Lower consumption of milk and dairy products adversely affects human health, as they contain many vitamins and minerals that have a beneficial effect on health.

The submitted paper deals with the issue of private labels in the segment of milk and dairy products, where we have focused on a selected group of customers, namely millennials. Our research aims to show how private labels function as an alternative to the purchase and which elements do have an influence in the case of a specific group of customers, so-called millennials.

Millennials, also known as Generation Y or Millennial generation are those born after 1980 and the first generation to come of age in the new millennium **(Polakevičová and Uhríková, 2015)**.

Scientific hypothesis

For a deeper analysis of the research objectives, the following hypotheses were formulated:

Hypothesis 1: We assume that there is a correlation between the purchase of private label products and the respondents´ gender.

Hypothesis 2: We assume that there is a correlation between the frequency of purchase of private labels in the category of milk and dairy products and the respondents´ gender.

Hypothesis 3: We assume that there is a correlation between whether the respondent is the end-user of the purchased private label products and his gender.

Hypothesis 4: We assume that there is a correlation between
what private labels evoke in the respondent and his gender.

Hypothesis 5: We assume that there is a correlation between the decisive factor in the purchase of private labels and the respondents´ gender.

Hypothesis 6: We assume that there is a correlation between what discourages respondents from the purchase of private labels and the respondents´ gender.

Hypothesis 7: We assume that there is a correlation between what would lead the respondents to the purchase of private label products and the respondents´ gender.

MATERIAL AND METHODOLOGY

This paper aimed to find out how Slovak millennials perceive private labels, in which categories they buy them, what motivates them and on the contrary, discourages them from the purchase, etc. An anonymous questionnaire survey was chosen as the main research method, in which a total of 549 respondents from all over Slovakia have participated (the sample of respondents can be considered as representative on the 95% confidence level and 5% error margin as n ≥384).

As it can be seen in Table 1, the majority of our respondents was represented by women (66.3% of respondents), students (67.94% of respondents), respondents with the first degree of higher education (39.2% of respondents), households with net family income over 1.501 € (25.87% of respondents) and city residents (62.84% of respondents).

The questionnaire survey consisted of a total of 16 questions formulated as closed ones with the possibility of one, respectively multiple responses, and four open

questions where respondents were free to express their opinion and six classification questions.

As the second research method, there was used the method of blind test, which was involved by 15 respondents from our focus group. In this test, respondents were offered to taste four samples of parenica – a traditional Slovak cheese as follows: one sample of a traditional brand, two samples of private labels, and one sample produced by a reeve. In this test, our respondents were asked to evaluate on a scale of 1 to 5, with 1 being the best and 5 the worst, the color, flavor, fragrance, and consistency of the given samples. Table 2 provides a more detailed overview of the examined parenica samples.

Table 1 Characteristics of Respondents.

Category of respondents	Number
Female	364
Male	185
Educational Structure of Respondents	**Number**
Primary education	23
Secondary education without A level	17
Secondary education with A level	226
University education- Bachelor degree	245
University education- Masters degree	36
University education- PhD. degree	2
Economic Activity of Respondents	**Number**
Student	373
Employed	147
Unemployed	7
Self-employed	13
Maternity leave	9
Net Money Income of Households per Month	**Number**
Up to 500 EUR	88
501 – 800 EUR	81
801 – 1.100 EUR	136
1.101 – 1.500 EUR	102
More than 1.501 EUR	142
Place of Residence of Respondents	**Number**
City	345
Countryside	204

Note: Source – results of the research.

Table 2 Exmined samples of parenica and their indication.

Indication of the sample	Examined parenica
Sample 1	Parenica produced by a reeve
Sample 2	Private label 1
Sample 3	Private label 2
Sample 4	Traditional brand

Note: Source – results of the research.

Statistical analysis

The results of the questionnaire survey and subsequently of the blind test were verified using statistical verification of formulated dependencies, using mainly the methods of Pearsons' Chi-Square Test, Phi Coefficient, Cramer´s V Coefficient and Friedman's test which were calculated in the statistical programs XL Stat and SAS Enterprise Guide. In hypothesis testing, if the p-value is lower than a significant level, in our case 0.05, the null hypothesis is rejected and its alternative is confirmed.

RESULTS AND DISCUSSION

Nielsen's recent global Consumer Confidence Index reports (2018) proved that there is a new retail revolution underway, and it's going to affect the food industry across the globe over the next five years in ways we have never seen before by the development of private-label products and the new challenges that this will present for brands and manufacturers across the globe, as retailers develop and market their products rather multinational name brands to meet changing consumer needs **(Herstein and Gamliel, 2004; Smith and Bashaw, 2009; Kakkos et al., 2015)**.

Private label brands, also known as "store brands" or "distributor brands", were considered low-price, low-quality products several decades ago; currently, however, they represent a clear alternative to manufacturer brands **(Kapferer, 2008)**. They account for more than 40% of the market in six European countries **(Private Label Manufacturers Association [PLMA], 2015)**. In general, private labels refer to brands owned by the retailer or distributor and sold only in its stores **(Kumar and Steenkamp, 2007)**. Conversely, manufacturer brands are brands owned by manufacturers to commercialize them.

Private labels are not new in the market, as firstly occurred at the end of the 19th century, **(Nagyová and Košičiarová, 2014)**. Nowadays, in a highly competitive environment, the private label products represent a suitable and perfect way of addressing new potential customers **(Polakevičová, 2015; Džupina et al., 2016; Mach et al., 2018; Lorincová et al., 2018; Balcarová et al., 2014**).

Consumers are currently in an increasingly competitive and dynamic market environment **(Mach et al., 2018; Balcarová et al., 2014)**, where the brand itself is either losing its weight or strengthening it. **Koprda (2014)**, **Džupina and Janková (2017)** as well as **Bulanda et al. (2018a)** emphasize that consumer behavior is a complex of behavior that is not influenced by just one factor. It is the combination of different factors and mainly of the price and quality.

Here can be seen the possibility for private labels, which can become a perfect alternative to traditional brands, bringing several benefits not only to the consumer and retailer but also to the supplier himself, especially in increasing his sales volume, lower communication and logistics costs, and options to enter new markets **(Corstjens and Lal, 2000; Collins-Dodd and Lindley, 2003; Richardson et al., 1996)**. While the main advantage for the consumer is the easy identification of private label products, the lower price, respectively the guarantee of authenticity, origin, and standard, as well as of comparable quality **(Tvrdoň and Přibyl, 2004)**, for the retailer it is the strengthening of image **(Liu and Wnag, 2008)**, expanding supply, increasing demand and strengthening customer loyalty **(Cheng et al., 2007; Huang et al., 2007; Kita et al., 2013)** as well as minimizing the risks associated with the introduction of new products **(Baltas, 1997; Sethuraman and Cole, 1999)** and consolidating its position in the retail market **(Lukić, 2011)**.

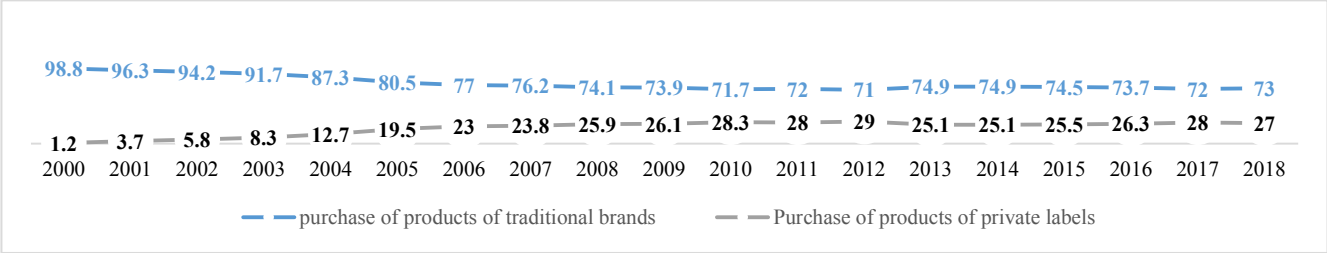


Figure 4 Percentage of purchases of branded products and products sold under private labels in Slovakia (%). Note: Source – own processing according to available sources.

Table 3 Results of Friedman's test (part a).

Friedman's test	
Q (Observed value)	620.449
Q (Critical value)	16.919
DF	9
p-value (Two-tailed)	<0.0001
alpha	0.05

Note: Source – results of the research.

The mentioned is also true for private labels, and especially those on the Slovak market, whose consumption has been steadily increasing, respectively is slightly fluctuating, as it can be seen from the Figure 4 – purchases of private labels by Slovak consumers have recently increased, respectively they have fluctuated, which in our opinion is largely due not only to the lower price of these products, but also to higher confidence in them, either their ever-increasing quality, which in many cases becomes not only comparable, but also higher than those of traditional brands.

The submitted paper focused on the issue of private labels in the segment of milk and dairy products in a selected segment of customers called millennials, because according to several researches **(Polakevičová and Uhríková, 2015; Šedík et al., 2018a; Šedík et al., 2018b)**, they represent potential customers, with a high potential to become loyal customers.

Submitted paper aimed was to find out how Slovak millennials perceive private labels, in which categories they buy them, what motivates them and on the contrary discourages them from the purchase, while we focused primarily on the segment of milk and dairy products regarding the fact, that many different studies proved that private labels are mainly purchased in the categories of milk and dairy products (e.g. research by **Košičiarová et al. (2014), Retailmagazin.sk (2018), GFK (2018), Košičiarová et al. (2018)** etc.) and the questions focused on the mentioned issue was formulated in the questionnaire survey.

In terms of purchasing private label products, the current situation between potential loyal customers and private label customers in Slovakia among Slovak millennials is favorable, as out of a total number of 549 respondents, 24.41% of respondents buy these products regularly and 57.92% of respondents buy them sporadically, while purchasing them mostly because of good quality, adequate price, good previous experience or recommendations from family and friends, the do not purchase them mainly because of their uninteresting package, low quality, high price as well as their lack of interest to try them. In the case of an exact frequency of purchase, it is possible to say that our respondents purchase private labels mainly once a month (31.06% of respondents), respectively multiple times a week (23.19% of respondents); buy mainly milk and dairy products followed by mineral waters, lemonades, and juices. Products milk and dairy private labels are purchased weekly (40.66% of respondents).

At least are private label products purchased in the

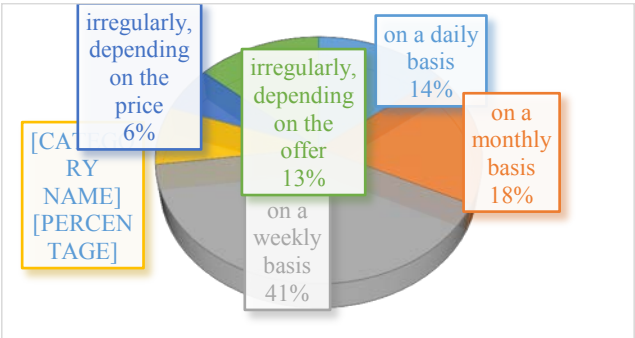


Figure 5 Frequency of purchase of milk and dairy products under the private label (%). Note: Source – results of the research.

Table 4 Results of Friedman's test (part b).

Sample	Frequency	Sum of ranks	Mean of ranks	Groups			
Milk and dairy products	549	2234.500	4.070	A			
Mineral waters, lemonades, juices	549	2487.500	4.531	A	B		
Sweets	549	2778.500	5.061		B	C	
Salty snacks	549	2793.000	5.087		B	C	
Meat and fishs	549	2931.000	5.339			C	
Coffee, tea	549	3028.500	5.516			C	
Deli	549	3078.500	5.607			C	
Frozen semi-finished products	549	3544.500	6.456				D
Ready meals	549	3602.500	6.562				D
Alcoholic drinks	549	3716.500	6.770				D

Note: Source – results of the research.

categories of frozen semi-finished products, prepared meals, and alcoholic beverages (Table 3 and Table 4).

Our results once again largely correspond to the results of previous researches, e.g. **TNS Slovakia (2015)**, **GFK Slovakia (2010)**, **Nagyová and Košičiarová (2014)**, **Košičiarová et al. (2017)**, etc. which show that Slovak consumers buy private labels primarily several times a week or once a week; they buy them mainly because of their cost-effectiveness, quality and confidence; and that every Slovak household has "favorite brands" in its regular and regular purchases **(TASR, 2010)**.

Because the consumer behavior is changing and nowadays to the forefront is becoming the healthy lifestyle, more people are suffering with special illnesses and specific needs, we have asked our respondents whether they purchase the specific categories of private label products, e.g. gluten-free products, low-fat products, etc. and if so, what are the reasons. The results of research proved, that Slovak millennials start to buy the specific categories of private label products, purchase them mainly sporadically (27.59% of respondents) or regularly (12.93% of respondents) and they do so mainly because of the following reasons: healthy lifestyle, need to try something new, reasonable price or due to the health reasons.

As the consumer is considered to be the end user or the consumer of the given product **(Bulanda et al., 2018b; Pilař et al., 2018)**, the questionnaire survey aimed at finding the answer to whether our respondents are the final users of purchased private label products. It can be confirmed that our respondents are the consumers of private label products, as they have stated that they are the final consumers of purchased private label products (up to 39.06% of the respondents) and therefore it is true that Slovak millennials represent potential loyal customers of private labels. Attracting customers is the primary goal of any business, as the customer creates a demand for goods and services and is very likely to become a loyal consumer who will become loyal to the given business or brand. In doing so, companies compete mainly by promoting and lowering prices to attract the largest customer base **(Kenton, 2018; Světlík and Bulanda, 2019; Kaliji et al., 2019)**.

From this it is necessary to realize what works in the given customer segment, respectively does not work. The results of our research show that while the decisive factor leading to the purchase of private labels is the combination of reasonable price and quality (47.72% of respondents), the decisive factor discouraging from the purchase of private labels is their price, low quality and lack of information about the exact manufacturer (20.04%, 17.12% and 13.84% of respondents). The mentioned results are also confirmed by the researches of e.g. **Kumar and Babu (2014) Paraffin, Zindove, and Chimonyo (2018)** and **Kurajdová et al. (2015)** confirm our results and identify quality and price as the main factors determining the purchase of specific products.

Up to the question, what would influence our respondents to the purchase of private labels, we can say that in the case of Slovak millennials it is mainly the recommendation from their families and friends (40.62% of respondents), resp. tastings (16.21% of respondents), free samples, or more interesting form of promotion (in both cases 15.12% of respondents).

It is very common and it can be said also misconception that the low price is also an indicator of poor quality **(Sproles, 1977; Völckner and Hofmann, 2007; Gabrielsen and Sørgard, 2007; Asker and Cantillon, 2010)**. Private labels are often characterized by a low price, which could mean lower quality. This is why, we have asked our respondents how they perceive private label products, what they suggest about them, what do they think about their quality, respectively whether they prefer them based their purchase and if so, in which product categories this happens.

The results of our research show that Slovak millennials perceive private labels as a suitable alternative to the purchase (58.29% of respondents), private labels evoke in them a sense of adequate quality at a reasonable price (59.74% of respondents), the quality of private labels is up to their opinion comparable with the quality of traditional brands (16.76% of respondents think so exactly and up to 42.08% of respondents think so rather), in the case of milk and dairy products they think that their quality is good or adequate (47.18% of respondents, Figure 6) up to 26.55% of respondents exactly prefer them in their purchase, and this is particularly the case of categories such as milk and dairy products, food in general and cosmetics.

The last questions in our questionnaire survey focused on the issue, whether our respondents would recommend private label products to the other consumers and what they would change on them, if they had that chance. According to our findings, young Slovak consumers would recommend private label products to other consumers (20.95% of respondents has declared the possibility of certainly yes and 46.99% respondents rather yes) and if they had the possibility, they would in particular increase the quality of private label products (28.96% of respondents), they would change their packaging and made it more attractive and lower their price (in both cases 15.49% of respondents).

Evaluation of tested dependencies

Hypothesis 1: We assume that there is a correlation between the purchase of private label products and the respondents´ gender – rejected.

Hypothesis 2: We assume that there is a correlation between the frequency of purchase of private labels in the category of milk and dairy products and the respondents´ gender – rejected.

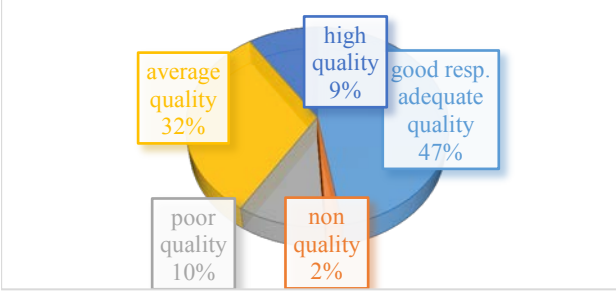


Figure 6 Perceived quality of milk and dairy products under the private label (%). Note: Source – results of the research.

Table 5 Correlation between whether the respondent is the end user of the purchased private label products and his gender.

Statistic	DF	Value	Prob
Chi-Square	3	20.8724	0.0001
Likelihood Ratio Chi-Square	3	21.2857	<.0001
Mantel-Haenszel Chi-Square	1	0.0424	0.8369
Phi Coefficient		0.1950	
Contingency Coefficient		0.1914	
Cramer's V		0.1950	

Note: Source – results of the research.

Table 6 Correlation between evoked stimulus regarding the private labels and the respondents´ gender.

Statistic	DF	Value	Prob
Chi-Square	2	7.7339	0.0209
Likelihood Ratio Chi-Square	2	7.5911	0.0225
Mantel-Haenszel Chi-Square	1	7.0413	0.0080
Phi Coefficient		0.1187	
Contingency Coefficient		0.1179	
Cramer's V		0.1187	

Note: Source – results of the research.

Table 7 Correlation between the decisive factor in the purchase of private labels and the respondents´ gender.

Statistic	DF	Value	Prob
Chi-Square	5	16.6665	0.0052
Likelihood Ratio Chi-Square	5	16.8441	0.0048
Mantel-Haenszel Chi-Square	1	0.8749	0.3496
Phi Coefficient		0.1742	
Contingency Coefficient		0.1716	
Cramer's V		0.1742	

Note: Source – results of the research.

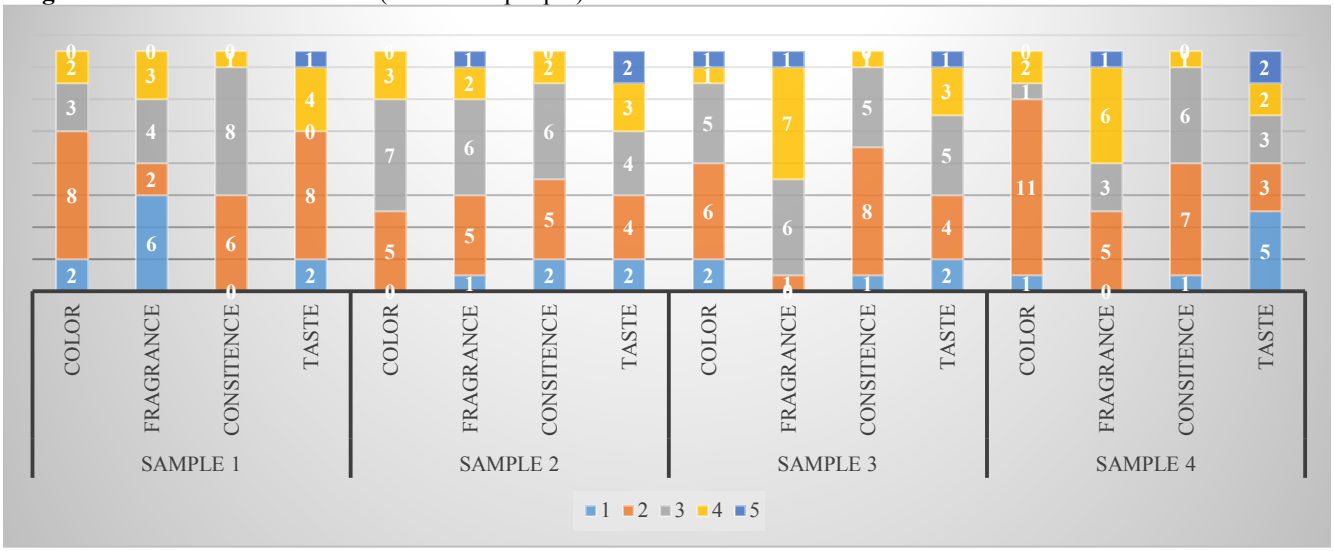
Hypothesis 3: We assume that there is a correlation between whether the respondent is the end-user of the purchased private label products and his gender – confirmed.
Hypothesis 4: We assume that there is a correlation between what private labels evoke in the respondent and his gender – confirmed.
Hypothesis 5: We assume that there is a correlation between the decisive factor in the purchase of private labels and the respondents´ gender – confirmed.
Hypothesis 6: We assume that there is a correlation between what discourages respondents from the purchase of private labels and the respondents´ gender – rejected.
Hypothesis 7: We assume that there is a correlation between what would lead the respondents to the purchase of private label products and the respondents´ gender – rejected.

In addition to the tested dependencies, it can be stated that although the dependencies 3, 4, and 5 were confirmed, they are weak but statistically still significant (Table 5 to 7).

Evaluation of the blind test

As it was mentioned in the part Material and Methodology, in the blind test, there were tested totally four samples of parenica – one sample of a traditional brand, two samples of a private label, and one sample produced by a reeve- on totally 15 respondents from our focus group (Table 2). Our respondents had to taste the mentioned samples without that, that they did not know which kind of parenica it is and they had at a rank from 1 to 5, were 1 is the best and 5 is the worst, evaluate their taste, color, fragrance, and smellAs it can be seen from the Figure 7, our results have proven that the best results are declared – in a case of parenica cheese produced by a reeve (best in the all aspects – in the case of color, consistency, and taste evaluated with the mark 2 and in the case of fragrance mark 1), resp. by a traditional brand (best mark in case of taste), but it must be also mentioned that the difference between the traditional brand and private labels is very small, because all of the tested samples have reached mainly the marks of 2.

Figure 7 Results of the blind test (number of people).



Note: Source – results of the research.

CONCLUSION

Consumer behavior, factors influencing the purchase, the decision-making process of consumers, etc. were of interest to several studies and research works, but very few of them have focused on millennials, not to mention private labels. The submitted paper focused on the issue of private labels but in a specific segment of customers called millennials, who represent potential loyal customers of private labels, and focused on the issue of private labels in the milk and dairy segment, as several researches has shown that private labels are mainly purchased in this category.

An anonymous questionnaire survey was chosen as the main research method, in which participated 549 respondents in the millennials category; which was supplemented by a blind test (15 respondents from the focus group). The results of our research point to many key findings in this issue, where it was proved that our respondents are indeed the end-users of purchased private label products, they buy them mainly in the category of milk and dairy products, their quality is comparable with the quality of traditional brands (also proven by the results of the blind test), that the decisive factor affecting the purchase of private label products is the combination of reasonable price and good quality, the decisive factor discouraging from the purchase of private labels is their price, low quality and lack of information about the exact manufacturer and what can lead our respondents to their purchase are primarily the recommendations of family and friends, and own experience (tastings and free samples).

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Zingiber cassumunar roxb. Extract increase the reactive oxidant level and interleukins expression *in vitro*

Nurkhasanah Mahfudh, Nanik Sulistyani, Ghina Adhila

ABSTRACT

Zingiber cassumunar Roxb. (bangle) has a variety of active compounds, including curcumin and phenylbutenoid. Bangle rhizoma reported exhibiting immunomodulatory activities. This research aims to determine the mechanism of bangle extract as an immunomodulator by the secretion of Reactive Oxygen Intermediate (ROI), Nitric Oxide (NO), and interleukin (IL-10 and IL-14) expression level. Bangle extract (*Zingiber cassumunar* Roxb.) was made by the maceration method using 96% ethanol solvent. This research was administered *in vitro* using macrophage cells from male mice with Balb/C strain divided into 2 groups: normal control and treatment group (receiving 25, 50, and 100 ppm of extract). The administration of bangle extract can function as an immunomodulator by an increase of ROI in 25 and 50 ppm of the extract significantly than the control group (p <0.05), the treatment groups decrease NO level (p <0.05), it also was found to increase expression of IL-10 and IL-14 expression levels (p <0.05). *Zingiber cassumunar Roxb.* extract was potentially to be developed as an immunomodulator.

Keywords: Immunomodulator; *Zingiber cassumunar* Roxb.; ROI; NO; IL-10; IL-14

INTRODUCTION

The immune system defends our body against invaders, such as viruses, bacteria, and foreign bodies which are the cause of various diseases. It consists of a natural immune system (innate immunity/non-specific) and an adaptive immune system (adaptive immunity/specific) **(Akrom, 2017; Baratawidjaja and Rengganis, 2014)**. Immunomodulators are pharmacological agents that can modulate a partial immune response that is spurred by an immune response, on the other hand, it inhibits some of the other immune system. Immunomodulators are restoring the imbalance of the disrupted immune system **(Akrom, 2017)**.

Macrophages are professional phagocytes that act as APC and the main effectors in cellular innate and adaptive immune response **(Murphy, 2012)**. In the body's defense mechanism against invaders, macrophages become the leading component of immune blocking. Macrophages express many surface receptors that can catch and swallow (degrade) microbes, in a process called phagocytosis **(Baratawidjaja and Rengganis, 2014)**. Phagocytosis and *reactive oxygen intermediates* (ROI) are the macrophages main mechanism in destroying infected cells **(Akrom et al., 2015)**.

Activate macrophages can stimulate the proliferation and activity of T and B lymphocyte cells. Macrophage cells act as antigen-presenting cells (APC) that will activate Th-0 lymphocytes. Activated CD4 Th-0 lymphocytes will proliferate towards Th-1 or Th-2 depending on the cytokine environment and the location of the antigen. Activated T lymphocytes will release various mediators, differentiation towards Th-1 will produce proinflammatory cytokines while differentiation towards Th-2 produces anti-inflammatory cytokines **(Thiery et al., 2003; Bastos et al., 2004; Akrom and Mustofa, 2017)**.

One of the potential plants in Indonesia is bangle (*Zingiber cassumunar* Roxb.) has been proven to have scientific activity as an immunomodulator. Bangle ethanol extract has been shown to have an immunomodulatory effect, indicated by an increase in the activity of macrophage cell phagocytosis, ROI secretion, and IL-10 expression, decreased NO secretion, and TNF-α production *in vivo* **(Arini et al., 2014; Nurkhasanah et al., 2017; Fitriana et al., 2018)**. *In vitro* research, phenylbutanoid compounds from bangle rhizomes can increase phagocytic activity of macrophage cells, and inhibit NO production **(Chairul et al., 2009; Nakamura et al., 2009; Kaewchoothong et al., 2012)**. Besides, the n-hexane bangle fraction can reduce the phagocytic activity of macrophage cells, and decrease lymphocyte proliferation **(Nurkhasanah et al., 2019b)**. In this study, we want to clarify the activity of bangle ethanol 96% extract as an immunomodulator by analyzing the secretion of ROI, NO, IL-10, and IL-14 by the macrophage.

Scientific hypothesis

Bangle has immunomodulatory activities with a mechanism of increasing ROI and NO secretion level, increasing IL-10 and IL-14 expression level, *in vitro*.

MATERIAL AND METHODOLOGY

Material and subject

Fresh *Z. cassumunar* rhizome purchased from the local market, Yogyakarta, Indonesia. The sample was verified and identified in the Biology Laboratory of Universitas Ahmad Dahlan. Macrophages were obtained from the peritoneal cavity of mice Balb/c strain aged eight weeks old (20 – 30 g) from the Integrated Research Laboratory of Universitas Gadjah Mada (Laboratorium Penelitian dan Pengujian Terpadu, LPPT UGM).

Research Procedure

Preparation of Bangle Extract

Z. cassumunar extraction was carried out using the maceration method and 96% ethanol as the solvent. The maceration was done for 3 x 24 hours. The macerate was filtered, and then evaporated (rotary evaporator) for 2 hours per day in a week, and used a water bath until a thick extract was obtained.

Preparation of Test Animals

The use of test animals in this research had received ethical approval from the Commission for Research Ethics of Universitas Ahmad Dahlan with Number: 011804063. The test animals were male mice Balb/c strain aged eight weeks old.

Isolation of Macrophages

Mice were narcotized with chloroform after being fasted for 10 – 12 hours. Then, mice were placed in the supine position. The mice's abdomen skin cleaned using disinfectant (70% alcohol) and dissected. The peritoneal sheath cleaned with 70% alcohol. Then, 10 mL of cold RPMI injected into the peritoneal cavity and shaken slowly for three minutes. The inner cavity pressed with two fingers and the fluid from the peritoneal cavity (the non-fatty part) drawn using an injection syringe to obtain an aspirate.

The aspirate is centrifuged at 1200 rpm, 4 °C for 10 minutes. The supernatant was removed, and the pellets (macrophages) resuspended with 1,000 µL complete medium. The number of cells counted from 10 µL macrophage suspension in a hemocytometer. The macrophage cell suspension grew in a 6-well microtiter plate (coverslip) with a density of 5 x 10^5 cells/well for the ROI and interleukin assay. And a 6-well microtiter plate with a density of 1 x 10^5 cells/well for the NO assay **(Ulfah et al., 2017; Nurkhasanah et al., 2017)**.

Reactive Oxygen Intermediate (ROI) Secretion Assay

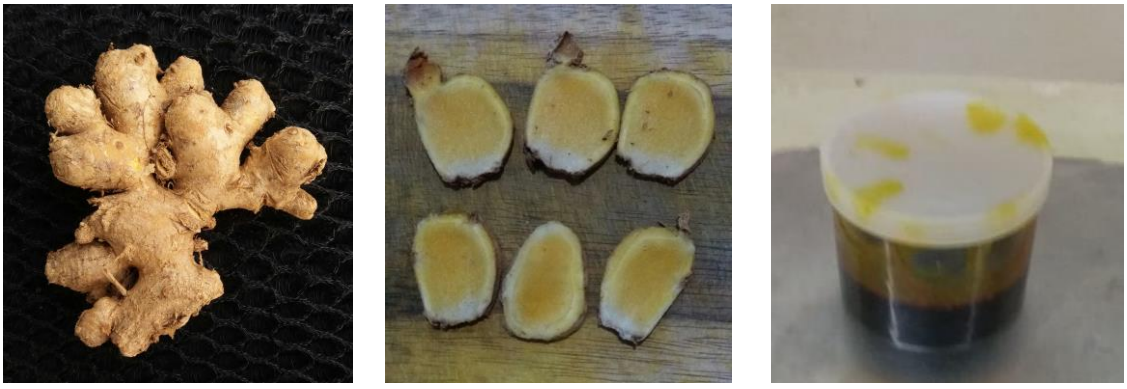
The 100 µL of macrophage cell suspension grew in a 6-well microtiter plate (coverslip) with a density of 5 x 10^5 cells/well. The cells incubated in a 5% CO_2 incubator at 37 °C for 15 minutes. 800 µL of complete RPMI medium added to each well, then microplates incubated overnight. The medium was removed and the sample added each well. The microplates were incubated in a 5% CO_2 incubator at 37 °C overnight.

The 50 – 100 µL of NBT solution and 1 mL of PBS (containing 125 PMA) added to each well, then incubated in 5% CO_2 incubator at 37 °C for 60 minutes. The reagent was removed, dried at room temperature, and fixed with absolute methanol. After dried, applied a 2% neutral red solution to the coverslip. The percentage NBT reduction of macrophage cells, it calculated from 100 cells examined by a binocular microscope (XSZ 107 BN, Novel) and Optilab with 400x magnification **(Nurkhasanah et al., 2017)**.

Nitric Oxide (NO) *Secretion Assay*

Griess Reaction Assay used for NO levels testing. Griess A solution prepared by dissolving 0.1 gram of N-(1-naphthyl) ethylene diamine hydrochloride (Sigma N, 5889) in 100 mL of distilled water. Griess B solution prepared by dissolving 1 gram of sulfanilamide (Sigma N 5589) in 100 mL of 5% orthiohisohoric acid (v/v). Both solutions stored at 0 – 4 °C protected from light. Standard nitrite prepared by dissolving 69.0 mg of sodium nitrite (Merck) in 100 mL of distilled water and stored at 0 – 4 °C protected from light. Stock standard solutions prepared using standard nitrite solutions in concentrations between 1.5625 – 100 µM **(Nurkhasanah et al., 2017)**.

100 µL macrophage cell suspension grew in 96-well microtiter plate-wells with a density of 1 x $10^5.mL^{-1}$. The samples were added to each well, and the microplates were incubated overnight. The standard nitrite was inserted into the blank section of the 96-well microtiter plate, to determine the standard curve. Each well added 50.0 µL



a **b** **c**

Figure 1 *Z. cassumunar* (a. rhizome; b. pieces of rhizome; c. extract).

Griess reagent, allow to stand for 5 – 10 minutes at room temperature and protected from direct light, until the color changes. The absorbance measured using an ELISA reader at a wavelength of 550 nm **(Nurkhasanah and Zulkarmen, 2014; Nurkhasanah et al., 2017)**.

Interleukin-10 and Interleukin-14 Expression Assay

Previously, the preparation of cell culture is the same as in ROI assay. Macrophage cells culture fixed using methanol and then washed with Phosphate Buffer Saline (PBS). The microplates soaked in 300 µL peroxidase blocking solution and washed with distilled water.

Then the microplates added 20 µL protein blocking serum, incubated at humid temperature for 10 – 15 minutes. Added 30 µL Interleukin-10 and Interleukin-14, incubated at room temperature then washed with PBS. Added with 30 µL of biotin, incubated at room temperature then washed with PBS. Added with 30 µL of the enzyme streptavidin-peroxidase, incubated at room temperature then washed with PBS. Added with 30 µL peroxidase substrate solution (DAB), incubated at room temperature, and washed with distilled water. Added with 100.0 µL of Mayer Hematoxylin (counterstain), incubated at room temperature then washed with distilled water. Then the microplates soaked in absolute alcohol, cleaned, and dried. The microplates dipped in xylol and dried. Then the microplates dropped with mounting media and covered using a deckglasser. After dried, observed in a binocular microscope (XSZ 107 BN, Novel) and Optilab with 400x magnification to examine the color of the cells, expression of IL-10 and IL-14 has intense brown **(Javois, 1999; Nurkhasanah et al., 2019a)**.

Statistic analysis

All statistical analyzes performed using the SPSS version 22 program. The normality test and homogeneity test performed about the data ROI levels, NO levels, and the expression of Interleukin-10 and Interleukin-14. Then proceed with the One-way ANOVA and LSD tests (with a significance level of 0.05).

The normality test performed using the Shapiro-Wilk‘s test, with total data of less than 50. The variant homogeneity test performed using the Levene‘s test. If the results of the normality test and homogeneity test are homogeneous variance and normally distributed, then the test continued with the analysis of one way ANOVA variants, and LSD test.

RESULTS AND DISCUSSION

Result of ROI Secretion Assay

The NBT reduction test (tetrazolium nitro blue reduction test, containing PMA (phorbol 12-myristate, 13-acetate)) was used to measure the ability of peritoneal macrophage cells to secrete ROI. NBT (formazan salt) will diffuse into cells, then tetrazolium succinate reductase enzyme will divide into formazan. ROI cause increased respiration and reduction of NBT by forming black formazan deposits **(Leijh et al., 1986)**. It can be seen in Figure 2, macrophage cells are black show secrete ROI due to formazan deposition.

In contrast to macrophage cells that do not secrete ROI, it looks only brown without any formazan deposits.

Table 1 shows the average levels of ROI secretion in the normal control group and the treatment group concentrations of 25, 50, 100 ppm.

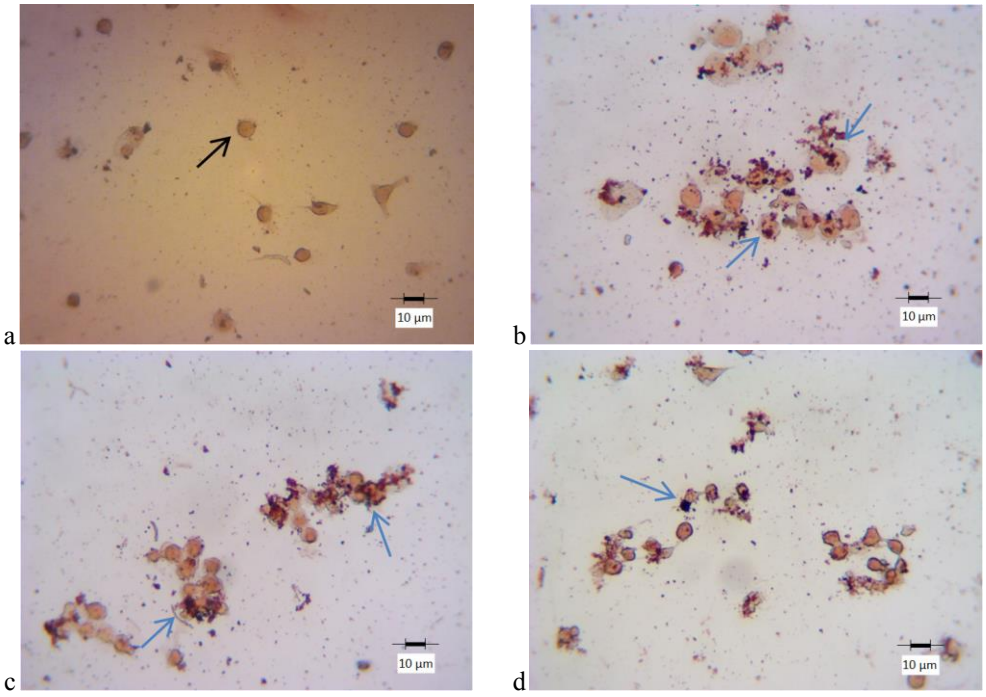


Figure 2 ROI secretion in macrophage cells after treated with bangle (Z. *cassumunar*) extract: a. normal control, b. bangle extract concentration of 25 ppm, c. bangle extract concentration of 50 ppm, d. bangle extract concentration of 100 ppm. (blue arrow: macrophage cells secrete ROI; black arrow: macrophage cells do not secrete ROI). Note: (400x magnification).

Table 1 ROI level by the administration of bangle (Z. *cassumunar*) extract.

Group	ROI secretion level (%) ±*SD*
Normal control	48.68 ±5.76
25 ppm	67.98 ±1.25*
50 ppm	70.38 ±8.35*
100 ppm	49.80 ±8.71[a,b]

Note: *significantly different compared to normal control group (p <0.05); [a]significantly different compared to concentration 25 ppm group (p <0.05); [b]significantly different compared to concentration 50 ppm group (p <0.05).

Table 2 NO level by the administration of bangle (Z. *cassumunar*) extract.

Group	NO secretion level (%) ±*SD*
Normal control	9.262 ±0.360
25 ppm	0.357 ±0.226*
50 ppm	0.762 ±0.840*
100 ppm	0.471 ±0.310*

Note: *significantly different compared to the normal control group (p <0.05).

The normal control group had the lowest ROI secretion (48.68%), not significantly different (p >0.05) with the treatment group concentration of 100 ppm (49.80%). The treatment groups concentrations of 25 and 50 ppm had ROI levels of 67.98% and 70.38%, significantly different (p <0.05) with normal controls, and the treatment group concentrations of 100 ppm.

There was an increase in ROI levels of the treatment group concentrations of 25 ppm and 50 ppm, but a decrease in ROI levels in the treatment group concentration of 100 ppm.

The content of curcumin compounds in bangle extract can cause increased levels of ROI. Curcumin can increase reactive oxygen species (ROS). This is related to macrophage activation and phagocytic activity of macrophages **(Mimche et al., 2011)**. Increased ROS can activate cellular signal pathways to form ROI **(Nathan and Ding, 2010)**. Bangle chloroform extract concentrations of 25, 50, and 100 $\mu g.mL^{-1}$ in vitro showed significantly increased ROI secretion compared to normal controls **(Nurkhasanah et al., 2019b)**. In vivo study, administration of bangle (5 $mg.20g^{-1}$ BW) by seven days duration can increase ROI secretion in mice induced by LPS (0.7 $mg.kg^{-1}$ BW) **(Nurkhasanah et al., 2017)**.

Phenylbutenoid is another component of the bangle extract and has anti-inflammatory activity by inhibiting enzyme cyclooxygenase-2 (COX-2) **(Jeenapongsa et al., 2003; Han et al., 2005; Leelarungrayub et al., 2017)**. The anti-inflammatory mechanism of this compound can be related to the ability of bangle to increase the expression of IL-10 **(Fitriana et al., 2018)**. Increased IL-10 expression will inhibit the production of IL-12, IL-1, and TNF-α. Inhibition of IL-1 and TNF-α production can affect T-cell activation to inhibit the inflammatory reaction. IL-12 has an important role in differentiating CD4 + into Th1 cells, then Th1 cells will secrete IFN-γ to activate macrophage cells to produce ROI. The inhibition of IL-12 production will indirectly inhibit the secretion of IFN-γ in case ROI production will decrease **(Bratawidjaja, 2014)**.

Result of NO Secretion Assay

NO is an effective antibacterial effector in the immune system. NO is a free radical synthesized by the enzyme nitric oxide synthase (NOS) through complex reactions. The main isoform expressed by macrophages is iNOS, this isoform will induce NO expression **(Kil et al., 2012)**. In this study, NO levels were measured using a Griess Reaction Assay (colorimetric method).

The concentration of NO secreted by macrophages will be calculated in the form of nitrites. Sulfanilamide (diazotization reagent) react with nitrite (in alkaline) will form to diazonium salt, then react with N-(1-naphthyl) ethylene diamine hydrochloride (coupling reagent) to be a stable form. The final result is intensive pink color and absorbance can be measured at wavelength 550 nm using Elisa Reader **(Nurkhasanah et al., 2017)**.

In Table 2 the average levels of NO secretion in the treatment group concentrations of 25, 50, and 100 ppm were 0.36; 0.76; and 0.47 μM. This result is significantly different (p <0.05) compared to the average NO level in the normal control group (9.26 μM). The decrease NO levels in the treatment group concentrations of 25, 50, and 100 ppm can be explained because of the results of IL-10 expression parameters. The results of IL-10 expression parameters are the treatment group 25, 50, 100 ppm has higher levels of IL-10 expression, and significantly different (p <0.05) compared to the normal control group.

The decrease NO levels were related to the results of the IL-10 parameter. In this study, the treatment group has a higher IL-10 expression than the normal group. iNOS gene expression is dependent on numerous proinflammatory cytokines in the cellular microenvironment of the macrophage, two of which include interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) **(Salim et al., 2016)**. IL-10 (macrophage inhibitor) acts to inhibit proinflammatory cytokine production included TNF-α, IL-1, and IL-12. IL-12 has a role to stimulate IFN-γ production. Over this explanation, the active component in bangle can act as an immunomodulator by reducing NO levels **(Goodyear-bruch and Pierce, 2002; Akrom, 2017)**.

Table 3 IL-10 expression of mice macrophages by the administration of bangle (*Z. cassumunar*) extract.

Group	IL-10 expression level (%) ±*SD*
Normal control	9.61 ±1.44
25 ppm	69.96 ±3.46*
50 ppm	69.26 ±2.98*
100 ppm	53.29 ±8.39*[a]

Note: *significantly different compared to normal control group (p <0.05); [a]significantly different compared to concentration 25 ppm group (p <0.05).

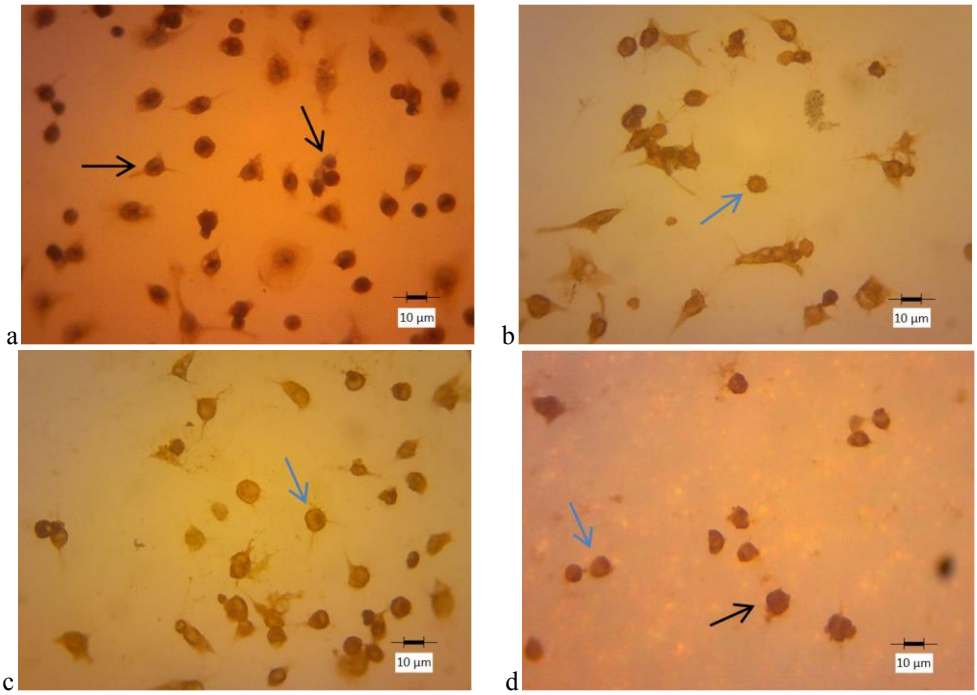


Figure 3 IL-10 expression on macrophage cells after being treated with bangle (*Z. cassumunar*) extract: a. normal control, b. bangle extract concentration of 25 ppm, c. bangle extract concentration of 50 ppm, d. bangle extract concentration of 100 ppm. (blue arrows: macrophage cells express IL-10; black arrows: macrophage cells do not express IL-10). Note: (400x magnification).

Phenylbutanoid was isolated from bangle (*Zingiber cassumunar* Roxb.) has an inhibitory effect of NO production on lipopolysaccharide-induced mouse macrophage cells (LPS) **(Nakamura et al., 2009)**. In other studies, *in vitro*, bangle can reduce NO secretion in murine macrophage RAW 264.7 cell lines **(Kaewchoothong et al., 2012)**. *In vivo* study, administration of bangle (5 mg/20g BW) can significantly reduce NO secretion in mice induced by LPS (0.7 $mg.kg^{-1}$ BW) **(Nurkhasanah et al., 2017)**. LPS causes an increase in NO levels of serum macrophages **(Tunctan et al., 1998)**. NO levels decrease after the administration of bangle extract could be due to the antioxidant content in the extract. Also, curcumin is another active component of bangle reported to inhibit NO production in macrophage activity **(Brouet and Ohshima, 1995)**.

Result of Interleukin Expression Assay

Interleukin-10

Observation of interleukin (IL) expression was carried out by an immunocytochemical method that uses specific antibodies to detect the expression of specific proteins (antigens) in cells. This research uses indirect immunocytochemical methods, the advantage is the results obtained have a more intense color, but it requires more time in the process **(Meshcer, 2017)**. The antigen will be bound indirectly to the primary antibody (IL-10 and IL-14) which has a role to recognize the antigen (first layer), then add a secondary antibody (biotin which binds to the enzyme streptavidin peroxidase) being the second layer.

The addition of secondary antibodies is also followed by the addition of chromogen substrate (DAB or 3,3-diaminobenzidine tetrahydrochloride), this substrate will be changed by enzymes so that it will form color deposits (pigments) in cells. To differentiate cells that are expressed IL-10 will have a brown color by DAB, while cells that are not expressed have a blue or purple color by Mayer hematoxylin (counterstain). Figure 3 shows the expression of IL-10 in macrophage cells treated with extract concentrations of 25, 50, and 100 ppm.

In Table 3 the average levels of IL-10 expression in the treatment group concentrations of 25, 50, and 100 ppm were 69.96%; 69.26%; and 53.29%. This result was higher and significantly different (p <0.05) compared to the average level of IL-10 expression in the normal control group (9.61%). The results obtained are suitable to those reported by other researchers that *Zingiber cassumunar* has the immunomodulatory activities one of activity by increase IL-10. This activity may be attributable to curcumin and phenylbutanoic as an active compound in this extract **(Fitriana et al., 2018; Nurkhasanah et al., 2020)**.

Table 4 IL-14 expression of mice macrophages by the administration of bangle (Z. *cassumunar*) extract.

Group	IL-14 expression level (%) ±SD
Normal control	2.16 ±0.30
25 ppm	87.44 ±7.35*
50 ppm	70.13 ±3.92*[a]
100 ppm	61.15 ±1.52*[ab]

Note: *significantly different compared to normal control group (p <0.05); [a]significantly different compared to concentration 25 ppm group (p <0.05); [b]significantly different compared to concentration 50 ppm group (p <0.05).

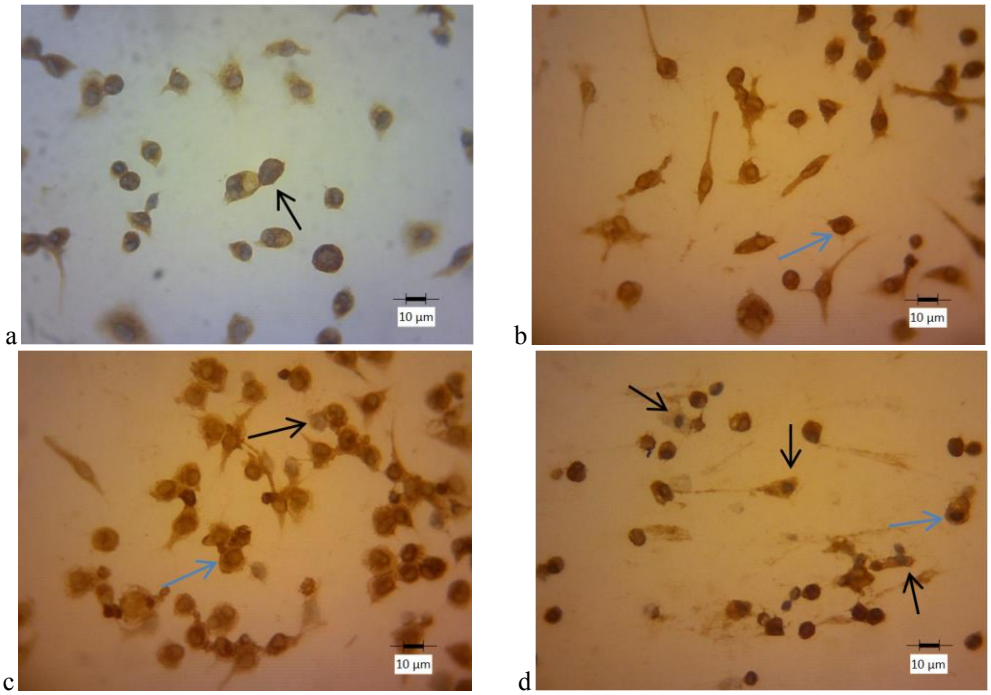


Figure 4 IL-14 expression on macrophage cells after being treated with bangle (Z. *cassumunar*) extract: a. normal control, b. bangle extract concentration of 25 ppm, c. bangle extract concentration of 50 ppm, d. bangle extract concentration of 100 ppm. (blue arrows: macrophage cells express IL-14; black arrows: macrophage cells do not express IL-14). Note: (400x magnification).

The treatment of bangle methanol fraction as a complementary therapy in mice infected with *P. berghei* can increase IL-10 levels **(Fitriana et al., 2018)**. It is known that the administration of bangle ethanol extract can inhibit the production of TNF-α which is a proinflammatory cytokine from Th1 cells, a decrease in TNF-α levels indicates an increase in IL-10 expression, where IL-10 (anti-inflammatory cytokines from Th cells - 2) can inhibit TNF-α production **(Perera et al., 2013; Arini et al., 2014)**. Also, there is a level of *in vivo* research with a length of 21 days and LPS stimulation of E. coli, 2,5 and 5 mg/20g BW of ethanol extract of bangle rhizome in mice can increase the expression of IL-10 **(Nurkhasanah et al., 2020)**.

Increasing the concentration of the test compound does not accord with an increased level of IL-10 expression because IL-10 is produced by active macrophages and Th-2 cells **(Akrom, 2017)**.

The results of the phagocytic activity parameters of macrophages also showed a decrease (%) of active phagocytic cells and phagocytosis index with an increase in the concentration of the test compound **(Adhila et al., 2019)**. The decrease in active macrophages will reduce the expression of IL-10 produced.

Interleukin-14

Similar to IL-10, the way to differentiate between cells expressed IL-14 will have a brown color by DAB, while cells that are not expressed have a blue or purple color by Mayer hematoxylin (counterstain). Figure 4 shows the expression of IL-14 in macrophage cells treated with extract concentrations of 25, 50, and 100 ppm.

In Table 4 the average levels of IL-14 expression in the treatment group concentrations of 25, 50, and 100 ppm were 87.44%; 70.13%; and 61.15%. This result was higher and significantly different (p <0.05) compared to the average level of IL-14 expression in the normal control group (2.16%). The administration of bangle ethanol extract showed the effect was to increase IL-14 expression. Although a decrease in IL-14 levels was seen with the administration the higher concentration of the extract. From this explanation, it can be seen that the active component of the bangle can act as an immunomodulator by improved IL-14 levels.

A previous study highlights that *Zingiber cassumunar* has the immunomodulatory activity which may be caused by curcumin and phenylbutanoic compound **(Chairul et al., 2009; Nurkhasanah et al., 2019b; Nurkhasanah et al., 2020)**. The ethyl acetate fraction of bangle extract concentration of 25, 50 and, 100 µg.mL^{-1} *in vitro* had a

role as an immunomodulator through increased IL-14 expression and the higher extract concentration showed the higher IL-14 expression produced **(Nurkhasanah et al., 2019b)**. Also, there is a level of *in vivo* research with a length of 21 days and LPS stimulation of E. coli, 5 mg.20g^{-1} BW of ethanol extract of bangle rhizome in mice can increase the expression of IL-14 **(Nurkhasanah et al., 2020)**.

CONCLUSION

Bangle ethanol 96% extract decreased NO levels (in all variations of extract concentration), and increased ROI levels compared to normal control groups (at extract concentrations of 25 and 50 ppm) with a significant effect (p <0.05). Also, bangle ethanol 96% extract increased in IL-10 and IL-14 levels compared to the normal control group (in all variations of the extract concentration) with a significant effect (p <0.05). These results indicate that bangle ethanol 96% extract has an immunomodulatory effect *in vitro*.

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PREVALENCE OF *CAMPYLOBACTER* SPP. IN A POULTRY AND PORK PROCESSING PLANTS

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ABSTRACT
The study aimed to investigate the prevalence of *Campylobacter* spp. in different stages of poultry and pork processing in the Central region of Russia. A total of 47 *Campylobacter* isolates were obtained from 107 samples from poultry processing plants (40.2%): 87.2% were identified as *Campylobacter jejuni,* whereas 12.8% were identified as *Campylobacter coli*. The prevalence of *Campylobacter* was significantly (p <0.05) higher after evisceration in the poultry processing plant. *Campylobacter* spp.was detected in 62.7% of the equipment and environmental samples. From positive samples of *Campylobacter spp.*, 84.3% of *Campylobacter jejuni,* and 15.7% *Campylobacter coli* were observed. A total of nine *Campylobacter* isolates were obtained from 116 samples from pork processing plants (7.8%): 33.3% of them were identified as *Campylobacter jejuni* whereas 66.7% were identified as *Campylobacter coli*. Splitting and evisceration were also critical in *Campylobacter* contamination. Almost all pork carcasses were *Campylobacter* positive, and all of them were identified as *Campylobacter coli*. The prevalence of positive *Campylobacter* samples in poultry processing plants was significantly (p <0.05) higher than in pork processing plants.

Keywords: *Campylobacter jejuni; Campylobacter coli*; poultry processing; pork processing

INTRODUCTION
Campylobacteriosis is still one of the most important infectious diseases that are likely to challenge global health in the years to come **(Kaakoush et al., 2015)**. According to the World Health Organization (WHO) reports, foodborne diseases, including Campylobacteriosis, are substantial: every year, almost one in 10 people fall ill and 33 million healthy life years are lost. *Campylobacter* is one of the four key global causes of diarrhoeal diseases **(WHO, 2020)**. The Centers for Disease Control and Prevention **(CDC, 2019)** estimates *Campylobacter* infection affects 1.5 million of the U.S. residents every year. Most cases are not part of recognized outbreaks, and more cases occur in summer than in winter **(EFSA and ECDC, 2019)**. The European Food Safety Authority (EFSA) reported that campylobacteriosis is the most common zoonotic disease in the EU. In 2018, member states reported 246,571 cases. The highest occurrence was detected in chicken meat (37.5%) and turkey meat (28.2%) **(EFSA and ECDC, 2019)**. Transmission typically occurs through the consumption of undercooked poultry or handling of raw poultry **(Altekruse et al., 1999; Blaser, 1997)**.

Studies have revealed that about 50% – 70% of human campylobacteriosis can be attributed to the consumption of poultry and poultry products **(Allos, 2001).** Various studies have demonstrated high levels of *Campylobacter* in the broilers, on the broiler carcasses, and retail chickens **(Zhao et al., 2001)**. Researchers have revealed this pathogen was detected in both dirty and clean transport crates, in scalding water, and on the de-feathering machine, and the working table at the end of the working day, but not at the beginning. After defeathering, *Campylobacter* spp. was detected in all of the sampled carcasses **(Perez-Arnedo and Gonzalez-Fandos, 2019)**. During slaughter, the main critical points for carcass contamination were identified as plucking, gutting, and final washing **(Facciolà et al., 2017)**. It was established that at low positive temperatures, *Campylobacter jejuni NCTC*11168 could remain viable in minced meat for at least seven days **(Bataeva and Sokolova, 2018)**.

However, in a study of goat and ovine milk in the Czech Republic, no *Campylobacter* bacteria were detected **(Bogdanovičová et al., 2015)**.

Campylobacter spp. survival was also investigated in the poultry industry before and after cleaning and disinfection. The fat removal machine, a gutting machine, a floor, a sink, a conveyor belt, shackles, and broiler meat were analyzed, and *C. jejuni* and *C. coli* were isolated. The results showed that the prevalence of *C. jejuni* and *C. coli* was 94.5% and 5.5%, respectively **(Sánchez et al., 2017)**. In one study, the detection of *Campylobacter* on carcasses was higher than that on cloacal swabs, which could

indicate cross-contamination during the slaughtering process **(Borges et al., 2020)**.

In some European countries, flock colonization of chickens with *Campylobacter* has a clear seasonal pattern, with the highest rates seen in the summer or autumn **(EFSA, 2010)**. The reasons for the seasonal variation are not fully understood but are likely to involve the frequency and nature of exposure of the flocks to *Campylobacter* spp. There is further evidence that climatic factors, such as temperature, correlate with both broiler flock and human infections **(Jorgensen et al., 2011)**.

Also, it has been reported that *Campylobacter* exhibits a cyclical pattern of contamination, where the level of contamination consistently increases and decreases depending on the season **(Hinton et al., 2004)**. Despite poultry are an important reservoir and source of human campylobacteriosis **(Hayama et al., 2011)**, the contribution of other sources, reservoirs, and transmission warrants further research. The predominant species in poultry is *C. jejuni*, whereas the predominant species of *Campylobacter* in pigs is *C. coli* **(Fosse et al., 2009; Horrocks et al., 2009; Varela et al., 2007)**. Authors also reported that control of this microorganism must rely on careful food processing and storage of pork, rather than an on-farm approach **(Varela et al., 2007)**.

Most human infections in the U.S. are associated with *C. jejuni*, whereas in Europe, a high incidence of human infection with *C. coli* is reported.

The authors reported that the sampling points with the greatest contamination rates were after evisceration, and contamination significantly decreased after chilling and washing **(Lee, et al., 2017)**.

Studies have shown that all processing plants sampled indicated a reduction in the *Campylobacter* populations along the processing line. Also, it was shown that proper cleaning of the equipment as well as a regular influx of freshwater, and using antimicrobials at the points of intervention during processing is crucial to preventing higher contamination **(Wideman et al., 2015; Berrang and Dickens, 2000)**.

Scientific hypothesis

This study was focused on the isolation of *Campylobacter* spp. from swabs of poultry and pork carcasses, and environmental swab samples from poultry and pork processing plants. The study aimed to investigate the prevalence of *Campylobacter* spp. in the processing of poultry and pork in Russian processing plants and to compare it with the European baseline data on Campylobacter prevalence.

MATERIAL AND METHODOLOGY

Poultry and pork processing plants in the Central region of Russia were selected. Swabs from poultry and pork carcasses and environmental swab samples from processing plants were selected as objects of the study. The following sampling points on the poultry processing line were selected: evisceration, processing and preparation, and packaging. The following sampling points on the pork processing line were selected: splitting and evisceration, removal of skin, deboning, and cutting.

Sampling

Environmental samples were taken using sterile sponges (3M TM, Saint Paul, 110 Minnesota, USA). Samples were transported at 4 °C to the laboratory and processed within 24 h.

Detection of *Campylobacter* spp.

The isolation of *Campylobacter* spp. was performed according to **ISO 10272-1 (2017)**. Environmental samples were performed according to **ISO 18593 (2018)**. They were taken using sterile sponges from 100 cm^2 and homogenized in 100 mL of Bolton broth (Merck, Germany). Swabs of poultry and pork carcasses were homogenized for 20 s with 225 mL of Bolton broth. The samples were incubated at 41.5 °C for 44 h under a microaerobic atmosphere. *Campylobacter* isolation was done on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Merck, Germany) and selective agar Preston under microaerobic conditions at 41.5 °C for 44 h. Confirmation of presumptive colonies was performed according to the **ISO 10272-1 (2017)** principles – typical colonies were seeded on blood agar (Oxoid, UK) and incubated at 41.5 °C for 24 h and then confirmed using biochemical tests (Oxoid, UK).

Statistical analysis

StatPlus 6.2.2.0 Software (AnalystSoft) was used. Tukey's test for the comparison of means was performed using the same program. The significance level was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Presence of Campylobacter spp. in environmental samples and poultry carcasses at various stages of poultry processing.

A total of 47 *Campylobacter* isolates were obtained from 107 environmental samples and poultry carcasses (40.2%): 87.2% were identified as *C. jejuni* whereas 12.8% were identified as *C. coli* (Figure 1).

Table 1 shows the presence of *Campylobacter* at different stages of poultry processing. After evisceration, *Campylobacter* spp. was detected in 62.7% of the equipment and environmental samples. From positive samples of *Campylobacter* spp. 84.3% of *C. jejuni* and 15.7% *C. coli* was observed. The predominance of *C. jejuni* over *C. coli* has been shown by other authors **(Sánchez et al., 2017)**. In that study, the abundances of *C. jejuni* and *C. coli* were 94.5% and 5.5%, respectively. These results confirmed those reported by **Lee et al. (2017)** that the greatest contamination rates were after evisceration. According to **Facciolà et al. (2017)** during slaughter, the main critical points for poultry carcass contamination were identified by plucking, gutting, and final washing. Other authors described slaughtering and evisceration as critical points of *Campylobacter* contamination **(Gruntar et al., 2015; Sasaki et al., 2013)**.

Campylobacter spp. was not detected after deboning and cutting, but it was found after packaging. The *Campylobacter* spp. isolated during packaging was identified as *C. jejuni*.

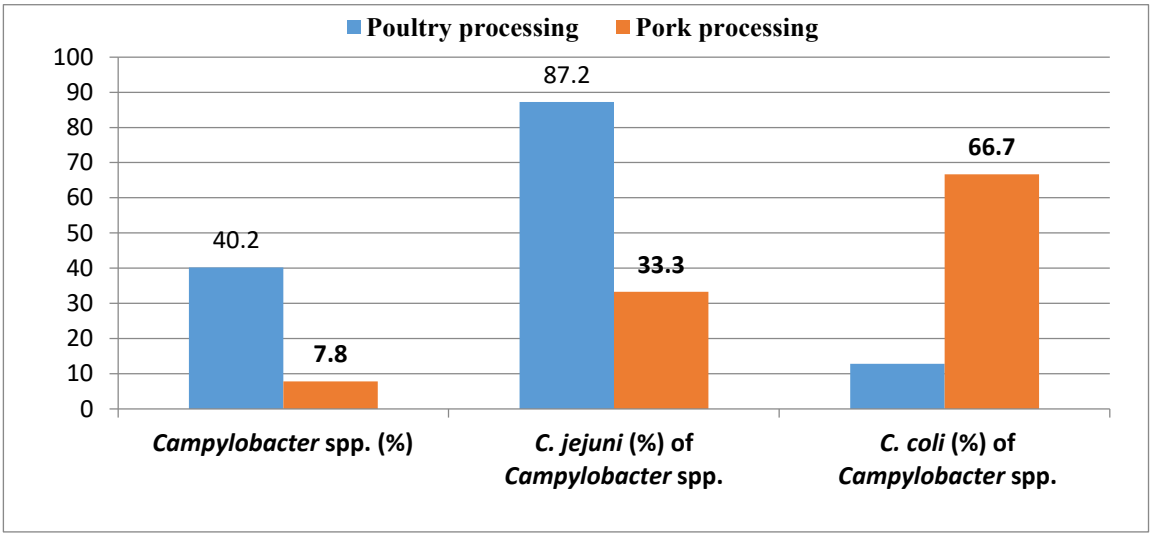

Figure 1 Prevalence of Campylobacter in poultry and pork processing plants.

Table 1 Presence of *Campylobacter spp.* in environmental samples and poultry carcasses at various stages of poultry processing.

Sampling location/Sample	*Campylobacter*/Total (%)	*C. jejuni*/Total positives (%)	*C. coli*/Total positives (%)
Evisceration	32/51 (62.7)	27/32 (84.3)	5/32 (15.7)
Bonning and cutting	0/9 (0.0)	0/0 (0.0)	0/0 (0.0)
Packaging	1/9 (11.0)	1/1 (100.0)	0/1 (0.0)
Poultry carcasses (total):	14/38 (36.8)	13/14 (93.0)	1/14 (7.0)
-cloaca	6/12 (50.0)	6/6 (100.0)	0/6 (0.0)
-legs	3/12 (25.0)	2/3 (67.0)	1/3 (33.0)
-carcasses	4/12 (33.0)	4/4 (100.0)	0/4 (0.0)
-neck	1/12 (8.3)	1/1 (100.0)	0/1 (0.0)

It is also an important contamination point due to the possible intestinal ruptures that can occur during the mechanical removal of the intestines **(Perez-Arnedo and Gonzalez-Fandos, 2019)**. Moreover, 50% of the investigated cloacal swabs samples were *Campylobacter* positive. These two stages can be related to each other and can cause cross-contamination of carcasses. Also, 5 mg of caecal content can increase the number of Campylobacter on eviscerated broiler carcasses **(Berrang et al., 2004)**. These findings support the idea of cross-contamination from contaminated equipment and work surfaces to carcass. Studies are confirming the genetic identity of the strains contaminating slaughterhouse equipment and meat products **(Elvers et al., 2011; Prachantasena et al., 2016)**.

Thirty-three percent of the investigated carcasses were *Campylobacter* positive. All *Campylobacte*r positive samples from cloacal swabs, carcasses, and necks were identified as *C. jejuni*.

However, in our research, the prevalence of *Campylobacter* was significantly (p <0.05) higher after evisceration than in carcasses. It is very important to decrease *Campylobacter* prevalence in poultry meat, because although *Campylobacter* spp. do not replicate in food **(Corry and Atabay, 2001)**, a low dose can cause an infection **(Vidal et al., 2014)**.

C. coli was detected in five environmental samples after evisceration and in the leg of one poultry sample.

Presence of Campylobacter spp. in environmental samples and pork carcasses at various stages of pork processing.

A total of nine *Campylobacter* isolates were obtained from 116 environmental samples and pork carcasses (7.8%): 33.3% of them were identified as *C. jejuni* whereas 66.7% were identified as *C. coli* (Figure 1). As reported in previous studies, *C. jejuni* prevailed in the poultry farm compared to the lower presence of *C. coli* **(Pepe et al., 2009; Peyrat et al., 2008; Wieczorek et al., 2015)**.

Table 2 shows the presence of *Campylobacter* at different stages of pork processing. After splitting and evisceration, *Campylobacter* spp. was detected in 7.4% of the equipment and environmental samples. A significant difference (p <0.05) in positive *Campylobacter* samples was found between poultry and pork evisceration. The prevalence of positive *Campylobacter* samples in poultry processing was significantly (p <0.05) higher than in pork processing. From two positive samples of *Campylobacter* spp, *C. jejuni* was observed. Environmental and equipment samples after removal of skin, deboning, and cutting were investigated. One of them was identified as *C. jejuni*, another one as *C. coli.*

Pork carcasses (neck, leg, belly, skin) were also investigated for the prevalence of *Campylobacter* spp. Almost all pork carcasses were *Campylobacter* positive, and all of them were identified as *C. coli*.

Table 2 Presence of Campylobacter spp. in environmental samples and pork carcasses at various stages of pork processing.

Sampling location/Sample	*Campylobacter*/Total (%)	*C. jejuni*/Total positives (%)	*C. coli*/Total positives (%)
Splitting and evisceration	2/27 (7.4)	2/2 (100)	0/2 (0.0)
Removal of skin	1/32 (3.1)	1/1 (100.0)	0/1 (0.0)
Bonning and cutting	1/21 (4.8)	0/1 (0.0)	1/1 (100.0)
Pork carcasses (total):	5/36 (13.9)	0/5 (0.0)	5/5 (100.0)
-neck	2/9 (22.2)	0/2 (0.0)	2/2 (100.0)
-leg	0/9 (0.0)	0/0 (0.0)	0/0 (0.0)
-belly	2/9 (22.2)	0/2 (0.0)	2/2 (100.0)
-skin	1/9 (11.1)	0/1 (0.0)	1/1 (100.0)

Our results confirm those reported by others, who found the predominant species of *Campylobacter* in pigs was *C. coli* **(Fosse et al., 2009, Horrocks et al., 2009; Varela et al., 2007)**. While the reservoirs of *Campylobacter* are recognised as both poultry and pigs **(Quintana-Hayashi and Thakur, 2012)**, *C. coli* is the main species found in pigs **(Avrain et al., 2004)**. Authors also reported that control of this microorganism must rely on careful food processing and storage of pork **(Varela et al., 2007)**. A factor that is associated with an increased risk of *Campylobacter* in pork is a high level of contamination in farms. Bacteriological study results showed that 77% of the piglets and 100% of the fattening pigs were infected with high levels of contamination, but *Campylobacter* was not detected after deboning **(Minvielle et al. 2007)**. The authors also note the importance of animal selection, transportation to the slaughterhouse, and time spent in the slaughterhouse **(Hald, Sommer and Skovgård ,2007)**.

The application of strict biosecurity measure proved to be effective in preventing the *Campylobacter spp.* contamination. There are: cleaning and disinfection of the plant equipment; a control of the entry of persons, birds, rodents or other animals; an insect control; water control; waste control **(Hansson et al., 2007; Guerin et al., 2007; Nesbit et al., 2001)**.

It was previously reported that survival during storage and under stress factors, such as microaerophilic conditions, *Campylobacter* in food products could be aerotolerant. Interestingly, a greater prevalence of aerotolerant strains (80%) was found among *C. coli* isolates as compared to *C. jejuni* isolates (6%); these strains were previously isolated from retail chicken meat, chicken livers, chicken gizzards, turkey, pork, and beef liver samples **(Karki et al., 2018)**.

Many studies describe the antibiotic resistance of *Campylobacter* strains **(Noormohamed and Fakhr, 2014)**. The increasing trend of antimicrobial resistance among *Campylobacter* strains indicates a high risk of new outbreaks **(Geissler et al., 2017)**.

Further studies are needed to investigate the antimicrobial resistance profile and aerotolerance of isolated *Campylobacter* strains. Potential approaches for the control of *Campylobacter* in processing poultry and pork plants are also necessary.

CONCLUSION

Campylobacter prevalence was estimated at poultry and pork processing plants in the Central Region of Russia. A total of 47 Campylobacter isolates were obtained from 107 samples of poultry processing (40.2%): 87.2% were identified as *C. jejuni,* whereas 12.8 % were identified as *C. coli*. The prevalence of *Campylobacter* was significantly (p <0.05) higher after evisceration in poultry processing plants: *Campylobacter spp.* was detected in 62.7% of the equipment and environmental samples. Of the positive samples of *Campylobacter* spp., 84.3% of *C. jejuni* and 15.7% *C. coli* were observed. A total of nine *Campylobacter* isolates were obtained from 116 samples of pork processing (7.8%): 33.3% of them were identified as *C. jejuni,* whereas 66.7% were identified as *C. coli*. Splitting and evisceration were a critical point of *Campylobacter* contamination. Almost all pork carcasses were *Campylobacter* positive, and all of them were identified as *C. coli*. The prevalence of positive *Campylobacter* samples in poultry processing was significantly (p <0.05) higher than in pork processing. The prevalence of *Campylobacter* was significantly (p <0.05) higher after evisceration in poultry processing plants: *Campylobacter spp.* was detected in 62.7% of the equipment and environmental samples. Among the positive samples of *Campylobacter* spp., 84.3% of *C. jejuni* and 15.7% *C. coli* was observed.

Further studies are needed to investigate the antimicrobial resistance profile and aerotolerance of isolated *Campylobacter* strains. Potential approaches for the control of *Campylobacter* in processing poultry and pork plants are also necessary.

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THE DONOR PROPERTIES OF RESOURCES RESISTANCE AGAINST THE EXCITER OF WHEAT RUST WHEAT

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ABSTRACT

A collection of soft winter wheat specimens investigated on the artificial infectious background of the leaf rust pathogen and selected resistance among them. The genetics of resistance sign in varieties and specimens were determined by hybridological analysis of F_2: Lovrin 32, KM 1485-6-8, VR 89 Bo 22, Beres, Tobarzo, 0-74-8-2, MIKM 1851-80, 4347-4, NS 326-99, 5517 A-5-5 Yr, Florida 302, VR 87 Bo 15, Matyo, NS 1308, 200-830, Polka, NS 2630/1, NS 18-30, HBE 0140-119, HBE 208-120, HBE 0303 156, HBE 0425-156, Tx91v4511, Tx92v4511, Plyska, Zernogradskaya 31, Volshebnitsa, Myronivska 40, Myronivska ostysta, Myronivska 28, Estet, Volynska napivintensivna, Kyivska 8, Expromt, Mironivska 29, Remeslivna, Garant, Selyanka, Erythrospermum 15761, Erythrospermum 12557, Erythrospermum 12735, Vympel odeskyiy during 1990–2018. The gene non-identity of the investigated donors was determined. In a variety of VR 89 Bo 22, 2 resistance genes, one of them *Lr19*, was investigated. The results of investigations of the composition of the leaf rust pathogen population by a series of isogenic lines and varieties of carriers of known effective resistance genes are presented. The high resistance against the leaf rust pathogen in the forest-steppe of Ukraine provide the genes *Lr9*, *Lr19*, *Lr37*, *Lr42* + *Lr24*, *Lr43* (*Lr21* + *Lr39*) + *Lr24*, *Lr9* + *Lr26*, *Lr10* + *Lr24*.

Keywords: wheat; gene pool; donor; infectious background; resistance genes

INTRODUCTION

Soft wheat is the leading grain crop by value in the world **(El-Khoury, 2009)**. The yield potential of this crop is not fully realized due to the damage to crops by phytopathogens. The diseases significantly reduce the yield and quality of the grain winter wheat. The gross collection losses are up to 20% annually, and in the epiphytotic years – 50% **(Novohatka, 1979)**. Breeding disease-resistant varieties are the most effective method in the struggle against wheat diseases **(Palamarchuk et al., 2019)**.

The creation of resistant varieties largely depends on the correct selection of initial material, determination of genetic factors of resistant control. However, the sources of resistance, the genetic nature sign of resistance to disease, is known identified not enough **(Dinh et al., 2020)** Therefore, the search and investigation of the immunological features of effective donors of resistance is the main problem in the creation of new competitive winter wheat varieties. Breeders need characteristic the varieties, not only by their phenotypic expression but also by their genetic characteristics recommended for hybridization **(Vyerchenko et al., 2019)**.

The leaf rust, the exciter of which is the fungus *Puccinia recondita* f. sp. *tritici*, is one of the most common diseases of wheat in Ukraine and the world. Breeding efficiency for leaf rust resistance can be improved by using different *Lr* resistance genes. More than 90 *Lr* genes are registered in the International Catalog Gene Symbol Directory, half of which are alien to date **(McIntosh, et al., 2003; McIntosh, et al., 2007; McIntosh, et al., 2008; McIntosh, et al., 2009; McIntosh, et al., 2010; McIntosh, et al., 2011; McIntosh, et al., 2012; McIntosh, et al., 2014; McIntosh, et al., 2016; McIntosh, et al., 2017; McIntosh, et al., 2018; McIntosh, et al., 2019)**. It is a great interest to investigate the resistance of wild relatives and endemic wheat species, identifying among them new immune forms and new effective resistance genes.

The immunity of *Triticum durum* and *T. dicoccum* must be widely used in the breeding of soft wheat for resistance to leaf rust **(Leonova et al., 2013)**. There are no reliable sources of resistance against this pathogen in the culture. But breeders are most interested in resistant varieties found among soft and durum wheat. World collections provide the possibility to use the achievements of breeding in different countries **(Kovalyshyna and Dmytrenko, 2017)**.

The investigation resistance against the disease of the gene pool of wheat is an important task, which can best be a solution from the attitude of the theory of co-evolution of

the plant and pathogen **(Mushtruk et al., 2020)**. Only based on such testimony can the right approach be made to the choice of sources of stability and the most effective method of breeding.

Scientific hypothesis

The scientific hypothesis is founded on identifying nature inheritance and manifest resistance genes to exciter of leaf rust. It is attaining by investigation of composition population exciter of disease and identifies resistance genes at collectible samples soft wheat. It is making it possible to increase the resistance gene pool and creating new heterogeneous varieties of soft wheat.

MATERIAL AND METHODOLOGY

The research material was winter wheat the collection specimens received from the World Collection Federal Center for Plant Genetic Resources, All-Russian Institute of Plant Genetic Resources, The Plant Production Institute named after V. Ya. Yuryev of NAAS and varieties of winter wheat from research institutions in Ukraine were materials for investigation.

The investigations were conducted under the conditions of artificial inoculation by the agent of leaf rust in the field infectious nursery of the Mironivka wheat institute named after V.M. Remeslo. A local and synthetic population of the pathogen obtained from the Institute of Plant Protection of the National Academy of Sciences of Ukraine was used for creating an artificial infectious background. The wheat plants contaminated with leaf rust spores in the field in the phase of plant exit into the tube according to the method of **(Bober et al., 2020)**. The variety of Mironivska 10 susceptible to this pathogen is used as an accumulator of infection in experiments. The resistance of plants against the disease was determined on a scale resistance of plants against the disease was determined on a scale **(Strahov, 1951)**, according to which the affection is expressed in relative percentages of leaf area covered with pustules of the pathogen **(Geshele, 1971)**.

Experiments on the evaluation of varieties and collections samples of wheat for disease resistance using artificial inoculation were laid out according to the schemes used in the system of state variety testing of crops **(Tkachyk, 2014)**, using the method developed by us to evaluate the resistance of wheat varieties against pathogens of major diseases **(Trybel et al., 2010)**.

The method of intraspecific hybridization, which was carried out by the "twell method" following the method used for creating the hybrid material of winter wheat **(Merezhko et al., 1973)**.

To identify resistance genes and the nature of the inheritance of the trait of resistance to the agent of leaf rust, using the method of hybridological analysis **(Radchenko and Odintsova, 2008)**. All samples were crossed with tester lines - carriers of known effective resistance genes MC Nair 2203 (*Lr9*), Flex (*Lr19*), Osage (*Lr24*) to identify effective resistance genes. Samples were crossed with each other according to an incomplete diallel scheme to determine the allelic ratio of genes.

Statistical analysis

To obtain information on the number and interaction of resistance genes, the obtained ratios of classes of resistant and susceptible plants (actual) were compared with one of the theoretically expected cleavages using the chi-square (χ^2) correspondence criterion.

The assumption that the difference between the actually obtained and theoretically expected splits is random was rejected if $\chi^2_{fact.}$ exceeded the critical $\chi^2_{st.}$ ($\chi^2_{0.05} = 3.84$). The error of results in statistical analysis $p = 0.05$. Statistical processing was performed in Microsoft Excel 2016 in combination with XLSTAT. Values were estimated using mean and standard deviations.

RESULTS AND DISCUSSION

The greatest immunological diversity of forms can be detected in the centers of co-origin of the host plant and the pathogen according to the coevolution theory of the host plant and the pathogen **(Leary et al., 2018)**. The center of origin of wheat is located in the Trans-Asian Center, which includes the Caucasus, which plays a leading role in it **(Dorofee, 1972)**. There is also a center for leaf rust formation **(Leppik, 1970)**.

In the work of scientists discovered the pattern in the distribution of immune forms among wheat species for the first time. He considered where there is a species or racial specialization of the pathogen, immune forms and varieties can be found. The Caucasus is the center of origin of many endemic, rust-resistant forms, and should occupy the first place in the globe in terms of the abundance of genetic and physiological types of wheat pointed **(Khaneghah et al., 2018)**. The *Triticum timopheevii* with complex resistance against all types of rust, powdery mildew, and soot was found in the Caucasus.

10 highly resistant endemic species of wheat and its relatives with the highest immunity to rust, in particular: *Triticum monococcum*, *T. timopheevii*, *T. militinae*, *T. Zhykovski*, *T. fungicidum*, *Haynatriticum*, *Aegilops umbellulata* are distinguished **(Varella et al., 2017)**.

In the work **(Casey et al., 2016)** found forms immune to rust, powdery mildew, and soot among *Triticum dicoccum*.

The principles of researching gene pool of wheat resistance against leaf rust, based on **(Flor, 1971)** theory of "gene-for-gene", developed **(Lodgering, Johnston and Hendricks, 1974; Berlyand-Kozhevnikov et al., 1985)**.

The majority number of leaf rust resistance genes identified in cultivated wheat varieties are derived from its wild relatives and endemic wheat species. The almost half of the known *Lr* genes are alien and transferred to *Triticum aestivum* from different types of wheat, egilops, couch grass, elimus according to the information given in the gene symbol catalogs **(McIntosh et al., 2003; McIntosh et al., 2007; McIntosh et al., 2008; McIntosh et al., 2009; McIntosh et al., 2010; McIntosh et al., 2011; McIntosh et al., 2012; McIntosh et al., 2014; McIntosh et al., 2016; McIntosh et al., 2017; McIntosh et al., 2018; McIntosh et al., 2019)**. The effective sources of resistance genes against the causative agent of leaf rust are *aegilops speltoides* – genes *Lr28, Lr35, Lr36, Lr47, Lr51, Lr66; Aegilops tauschii – Lr4, Lr21, Lr22a, Lr32, Lr39, Lr42; Aegilops umbellulata – Lr9, Lr76; Aegilops triuncialis – Lr58, LrTr; Aegilops ventricosa – Lr37; Aegilops kotschyi – Lr54; Aegilops sharonensis – Lr 56;*

Aegilops peregrine – Lr59; Aegilops neglecta – Lr62; Aegilops geniculate – Lr57; Elymus trachycaulis – Lr55; Secale cereale – Lr25, Lr26, Lr45; Thinopyrum elongatum – Lr19, Lr29, Lr24; Triticum timopheevii – Lr18, Lr50; Triticum spelta – Lr44, Lr65, Lr71; Triticum dicoccoides – Lr53, Lr64; Triticum timopheevii spp. *viticulosum – LrTt1; Triticum turgidum – Lr61;Triticum monococcum – Lr63.* All found effective resistance genes except *Lr10* and *Lr23* are alien.

The new highly efficient resistance genes against the local leaf rust population: *LrAc1*, *LrAc2* – from *Aegilops cylindrical*, *LrTe1*, *LrTe2* – from *Triticum erebuni* and *LrAd1*, *LrAd2* – from amphidiploid Ad4 (*Triticum dicoccoides* x *Triticum tauschii*) are identified by **Babaiants (2011)**. The collection samples obtained from the National Center for Plant Genetic Resources of Ukraine, varieties of different breeding establishments of Ukraine, and Mironivka Institute of Wheat named after V. M. Remeslo were investigated to select sources of resistance to leaf rust under conditions infectious background of the pathogen on a specially assigned phytoplot.

The effective resistance genes for the Forest Steppe zone of Ukraine leaves: *Lr9*, *Lr19*, *Lr24*, *Lr25*, *Lr37*, *Lr42* + *Lr24*, *Lr43* (*Lr21* + *Lr39*) + *Lr24*. It was installed as result investigations of the composition population leaf rust on a series of isogenic lines of Thatcher variety and varieties of carriers of known effective resistance genes (Table 1-2).

There was a slight lesion of *Lr19* gene carrier, indicating that clones were virulent against it. In some years, there was a slight lesion of the pathogen and on varieties protected by the *Lr9* gene. The varieties protected by the *Lr24* gene lose their stability. The *Lr37*, *Lr42* + *Lr24*, *Lr43* (*Lr21* + *Lr39*) + *Lr24*, *Lr9* + *Lr26*, *Lr10* + *Lr24*, *Lr19* + *Lr25* genes have shown high efficiency in recent years.

Genes show the high efficiency against the local population of the brown rust pathogen in the Forest-steppe of Ukraine: *Lr9*, *Lr19*, *Lr37*, *Lr42* + *Lr24*, *Lr43* (*Lr21* + *Lr39*) + *Lr24*, *Lr9* + *Lr26*, *Lr10* + *Lr24* **(Kovalyshyna, 2013)**, *Lr24*, *Lr25*, *Lr28*, *Lr34*, *Lr36*, *Lr41*, *Lr42* are determined by results of investigations domestic researchers **(Lisova, 2012)**. The virulence genes against *Lr9*, *Lr19*, *LrAc1*, *LrAc2*, *LrTe1*, *LrTe2*, *LrAd1*, *LrAd2* are rarely found in the Steppe of Ukraine **(Babaiants, 2011)**.

The genetics of the trait resistance of these samples was able to determine by hybrid analysis of F_2 (Table 3). The resistance of variety the Lovrin 32, KM 1485-6-8, VR 89 Bo 22, Beres, Tobarzo, Mironivska 40, Mironivska ostysta, Estet, Mironivskay 28, Volynska napivintensivna, Kyivska 8 is controlled by two dominant genes (15 : 1) (confidence probability p = 0.95). The resistance to the agent of leaf rust is controlled by two genes, one of which is dominant and one is recessive (13 : 3) in varieties 0-74-8-2, MIKM 1851-80, 4347-4, NS 326-99, Pliska, 5517 A-5-5 Yr, Florida 302, VR 87 Bo 15, Matyo, HBE 0140-119, HBE 208-120, Expromt, Mironivska 29, Remeslivna, Garant, Selyanka (confidence probability p = 0.95). Two complementary genes (9 : 7) control resistance in samples NS 1308, 200-830, Polka, Zernogradskaya 31, Volshebnitsa, Vympel odeskyiy, Erythrospermum 15761 (confidence probability p = 0.95). Two duplicate recessive resistance genes (7 : 9) identified in samples NS 2630/1,

Table 1 Affection exciter leaf rust *Lr*-line Thatcher series of soft spring wheat (Mironivskyiy Institute of Wheat named after V. M. Remeslo, NAAS, 2006 – 2018).

The name of the line	Pedigree	Gene of resistance	Origin	The intensity of the affection, %			
				2006 – 2010	2011 – 2015	2016	2018
TcLr1	Thatcher*6/Centenario	*Lr1*	CAN	34.0	27.5	3.0	20.0
TcLr2a	Thatcher*6/Webster	*Lr2a*	CAN	38.0	18.5	5.0	15.0
TcLr2c	Thatcher*6/Brevit	*Lr2c*	CAN	42.0	26.5	5.0	10.0
TcLr3	Thatcher*6/Democrat	*Lr3*	CAN	42.0	27.5	7.0	15.0
TcLr3bg	Thatcher*6/Bage	*Lr3bg*	CAN	40.0	22.5	5.0	10.0
TcLr3ka	Thatcher*6/ Klein Aniversario	*Lr3ka*	CAN	40.0	32.5	7.0	5.0
TcLr9	Thatcher*6/*Ae.umbellu*	*Lr9*	CAN	0.4	0	0	0
TcLr10	Thatcher*6/Lee	*Lr10*	CAN	29.0	20.0	7.0	10.0
TcLr11	Thatcher*6/Hussar	*Lr11*	CAN	21.5	20.0	6.0	10.0
TcLr12	Thatcher*6/Exchange	*Lr12*	CAN	14.0	7.5	7.0	7.0
TcLr13	Thatcher*6/Frontana	*Lr13*	CAN	12.0	6.5	3.0	3.0
TcLr14a	Thatcher*6/Hope	*Lr14a*	CAN	21.5	25.0	10.0	15.0
TcLr14b	Thatcher*6/Bowie	*Lr14b*	CAN	37.0	22.5	10.0	15.0
TcLr19	Thatcher*6/*Agropiron elongatum*	*Lr19*	CAN	0.4	0	0	0
TcLr23	Thatcher*6/Gabo	*Lr23*	CAN	28.0	11.5	3.0	5.0
TcLr24	Thatcher*6/ *Agropiron elongatum*	*Lr24*	CAN	5.0	10.0	1.0	1.0
TcLr25	Thatcher*6/Rosen (*Secalecereale*)	*Lr25*	CAN	2.5	1.5	0	0
TcLr26	Thatcher*6/Imperial (*Secalecereale*)	*Lr26*	CAN	21.0	27.5	10.0	15.0
TcLr29	Thatcher*6/ *Agropiron elongatum*	*Lr29*	CAN	34.0	15.0	5.0	10.0
TcLr30	Thatcher*6/Terenzio	*Lr30*	CAN	36.0	25.0	3.0	15.0
TcLr32	Thatcher*6/*Aegilops taushii*	*Lr32*	CAN	33.0	36.0	5.0	10.0
TcLr34	Thatcher*6/Terenzio	*Lr34*	CAN	33.0	15.0	3.0	5.0
Leaf Rust Monogene Line ECH		*LrEch*	CAN	26.0	25.0	1.0	1.0
Myronivska 10 (standart susceptibility)		–	UKR	45.0	58.7	30.0	60.0

Note: *In 2017, no research was conducted.

Table 2 Characteristics of soft winter wheat collection samples with known *Lr* genes for resistance to leaf rust (Mironivskyiy Institute of Wheat named after V. M. Remeslo, NAAS, 2006 – 2018).

No	The name of the line	Gene of resistance	Origin	The intensity of the affection, %			
				2006 – 2010	2011 – 2015	2016	2018
1	Arthur 71	*Lr9*	USA	0	0.02	3.0	0
2	Mc Nair 2203	*Lr9*	USA	0	0	1.0	0
3	Agrus	Lr19	USA	0	0.1	1.0	1.0
4	Flex	*Lr19*	USA	1.8	0.2	1.0	0
5	V1275	*Lr19*	FRA	0.1	0.2	0	1.0
6	VR 89 Bo 22	*Lr19*	FRA	0.2	0.2	0	1.0
7	Frederik	*Lr23*	CAN	3.0	7.0	5.0	5.0
8	Osage	*Lr24*	USA	3.9	3.0	1.0	2.0
9	Blueboy II	*Lr10+ Lr24*	USA	0.8	2.8	1.0	2.0
10	Transfer	*Lr19+ Lr25*	USA	0.8	0.6	0	0
11	Rendezvous	*Lr37*	GBR	0	0	3.0	1.0
12	203-238	*Lr9+ Lr26*	BGR	0	0.5	0	1.0
13	Century	*Lr24+ Lr42*	USA	0	0	0	0
14	TAM-200	*Lr24+ Lr21 + Lr39*	USA	0	0	0	0
15	Myronivska 10 (standard susceptibility)	–	UKR	45.0	45.0	30.0	60.0

Note: *In 2017, no research was conducted.

Table 3 Genetic characteristics of leaf rust resistance donors (Mironivskyiy Institute of Wheat named after V. M. Remeslo, NAAS, 1990 – 2018).

Resistance donors	Cleavege by phetype (resistant: susceptible)	The nature of interaction genes
Lovrin 32, Myronivska 40, Myronivska ostysta, KM 1485-6-8, VR 89 Bo 22, Myronivska 28, Estet, Volynska napivintensyvna, Kyiivska 8, Beres, Tobarzo 0-74-8-2, MIKM 1851-80, 4347-4, Plyska, NS 326-99, 5517 A-5-5 Yp, Florida 302, VR 87 Bo 15, Expromt, Myronivska 29, Remeslivna, Garant, Selyanka, Matyo, HBE 0140-119, HBE 208-120	15: 1 9 *A-B-* : 3 *A-bb* :3 *aaB-* : 1 *aabb*	Genes *A* and *B* are manifesting equally. Resistance dominates in both cases (two duplicates dominance genes)
	13: 3 9 *A-B-* : 3 *A-bb* : 3 *aaB-*: 1 *aabb*	Genes *A* and *B* are manifesting equally. Resistance dominates in case *AA* and recessive *bb* (two duplicates genes, one dominance and one recessive)
NS 1308, Erytrospermum 15761, 200-830, Vympel odeskyii, Zernogradskaya 31, Volshebnitsa, Polka	9: 7 9 *A-B-* :3 *A-bb* : 3 *aaB-* : 1 *aabb*	Genes *A* and *B* are interacting complementary. The presence of *A* or *B* alone is not sufficient to demonstrate the resistance of the phenotype (complementation of two dominant genes)
Erytrospermum 12557, Erytrospermum 12735, NS 2630/1, NS 18-30, Tx 91 v 4511	7: 9 9 *A-B-* : 3 *A-bb* : 3 *aaB-* : 1 *aabb*	Genes *a* and *b* are manifested equally. Recessive resistance in both cases (two duplicate recessive genes)
HBE 0303 156, HBE 0425-156	3: 1 1 *A* : 2 *Aa* : 1 *aa*	One dominant gene
Tx 92 v 4511	1 : 3 1 AA : 2 Aa : 1 aa	One recessive gene

NS 18-30, Tx 91v4511, Erythrospermum 12557, Erythrospermum 12735 (confidence probability $p = 0.95$). One dominant resistance gene (3 : 1) contains samples HBE 0303 156, HBE 0425-156, and the collection sample Tx 92v4511 contains one recessive resistance gene against leaf rust (1 : 3) (confidence probability $p = 0.95$).

The splitting observed, by crossing sources of resistance among themselves in all combinations of F_2, which confirms the non-identity of genes in these donors. The crossing of resistance donors with testers of known efficient genes *Lr9*, *Lr19*, *Lr24* observed splitting into resistance and unresistance except for the combination of VR 89 Bo 22 x Flex (*Lr19*). In ones, splitting into resistance and unresistance phenotypes was not detected. This indicates that VR 89 Bo 22 contains the *Lr19* genes (confidence probability $p = 0.95$).

The genetic control of the resistance sign in 42 donors was investigated. The variety VR 89 Bo 22 contains the

Lr19 genes. Other donors of this sign have resistance genes independent of the known effective ones, which makes it possible to replenish the leaf rust resistance gene bank and create new heterogeneous winter wheat varieties on this basis.

Successful breeding requires a clear understanding of the evolution of the pathogen and host-pathogen interactions, of the types of resistance, methods of material evaluation, resistance genes, and the nature of inheritance of this trait and its relationship to other economic and biological traits. It is only based on such testimony that the right approach to the choice of sources of resistance and the most effective method of breeding work can be worked out.

CONCLUSION

The more than 200 collections samples and varieties of winter wheat of different ecological and geographical origins were evaluated for resistance to leaf rust on the artificial infectious background of its pathogen during 1990 – 2018. 42 samples were identified that examined the genetics of resistance to the disease among them. The samples: Lovrin 32, KM 1485-6-8, VR 89 Bo 22, Beres, Tobarzo, 0-74-8-2, MIKM 1851-80, 4347-4, NS 326-99, 5517 A-5-5Yr, Florida 302, VR87Bo15, Matyo, NS 1308, 200-830, Polka, NS 2630/1, NS 18-30, HBE 0140-119, HBE 208-120, HBE 0303 156, HBE 0425-156, Tx91v4511, Tx92v4511, Plyska, Zernogradskaya 31, Volshebnitsa, Myronivska 40, Myronivska ostysta, Myronivska 28, Estet, Volynska napivintensivna, Kyivska 8, Expromt, Myronivska 29, Remrslivna, Garant, Selyaynka, Erythrospermum 15761, Erythrospermum 12557, Erythrospermum 12735, Vympel odeskyiy contain resistance genes are independent of each other and known to be effective (confidence probability $p = 0.95$). In the variety VR 89 Bo 22 one of the genes *Lr19*. This makes it possible to replenish the leaf rust resistance gene bank and create new heterogeneous varieties of winter wheat.

As a result of studying the composition of the leaf rust population on a series of isogenic lines of Thatcher variety and varieties of carriers of known effective resistance genes, we determined that the resistance genes of the forest-steppe zone of Ukraine remain: *Lr9*, *Lr19*, *Lr24*, *Lr25*, *Lr37*, *Lr42+Lr24*, *Lr43* (*Lr21* + *Lr39*) + *Lr24*.

As a result of research, it was found that the wheat variety Myronivska Ukrainian selection is quite productive in comparison with other varieties and resistant to pathogens of various diseases, especially to the pathogen (brown rust).

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EFFECTS OF MEAT AND PROCESSED MEAT CONSUMPTION ON THE LIPID PROFILE IN THE POPULATION WITH CARDIOVASCULAR DISEASES

Jana Kopčeková, Jana Mrázová, Martina Gažarová, Marta Habánová

ABSTRACT

Meat represents an important source of high-quality dietary protein for a large proportion of the global population. Also, red meat, in particular, significantly contributes to the intake of a wide range of micronutrients, including iron, zinc, selenium, vitamin D, and vitamin B12. Excessive consumption of meat and meat products is often associated with overconsumption of energy and fat, resulting in excess weight, obesity, and an increased risk of chronic diseases, such as cardiovascular disease and type 2 diabetes. This study aims to evaluate the relationship between meat and processed meat consumption frequencies and lipid profile in a group of 800 randomly selected patients hospitalized in the Cardiocentre Nitra. Patients were 20 – 101 years, (men, the average age was 61.13 ±10.47 years). The data necessary for the detection of dietary habits were obtained by a questionnaire method. Statistical comparisons between groups were made utilizing a one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. Our results show, that most respondents consume meat 1 – 2 times per week, while we did not notice a significant effect (p >0.05) of the type of meat on the lipid profile. The highest T-C, LDL-C, and TG values were seen in men who consume pork 3 – 4 times per week. Statistically significant was only the effect of pork meat on total cholesterol and triglycerides (p <0.05). In the consumption of beef and poultry, there was a non-significant effect on biochemical parameters of blood (p >0.05). We recorded a significant effect (p <0.05) of the consumption of frankfurters between consumption 1 – 2 times per week and 3 – 4 times per week. Up to 40.2% of respondents consume salami 3 – 4 times per week, and we recorded a significant effect on LDL levels between consumption 1 – 2 times per week and sometimes (p <0.05). Respondents who consume sausage, headcheese, and others products 1 – 2 times a week have non-significant higher T-C, LDL, TG, and lower HDL compared to less frequent consumption. High consumption of meat, mainly pork and processed meat seems to be associated with higher levels of total cholesterol, LDL cholesterol, and triglycerides.

Keywords: meat; processed meat; cardiovascular disease; lipid profile; dietary habits

INTRODUCTION

The incidence of cardiovascular diseases (CVD) is rapidly increasing worldwide and is currently considered to be the leading cause of death in both developing and developed countries **(Gaziano et al., 2010; Mittal and Singh, 2010)**.

Nutrition is widely recognized as a crucial driver of chronic disease **(Mozaffarian, 2016)**. Dietary habits influence many risk factors for cardiometabolic health, leading to type 2 diabetes, stroke, and heart disease, which are among the leading causes of death globally. Collectively, these risk factors associated with poor quality diet pose substantial health and economic burdens, and studies have shown that dietary factors are one of the main causes of the global burden of disease (measured as disability-adjusted life years) **(GBD 2016 Risk Factors Collaborators, 2016)**.

Most people all over the world eat meat, and meat is central to Western diets **(Pfeiler and Egloff, 2018)**. Consumption of red meats (meats of mammalian origin including beef, pork, and lamb) and processed meats (meats transformed through salting, curing, fermentation, smoking, or other processes to enhance flavor or improve preservation) has been increasing rapidly worldwide **(Godfray et al., 2018; Willett et al., 2019)**.

The epidemiologic literature usually classifies the meat consumed as ”red”, ”white”, and processed meat. Although it does not exist a clear classification of meat and subtypes, in general, all meats obtained from mammals are red meats because they contain more myoglobin than white meat (obtained from chicken or fish) **(Clonan, Roberts and Holdsworth, 2016)**. Processed meat refers to any meat that has been transformed through one or several of the following processes: salting, curing, fermentation, smoking, or other processes to enhance flavor or improve preservation. Most processed meats are made from pork or beef, but may also include other red meats, poultry, offal, or meat by-products such as blood. It is also important to

distinguish between industrial processing and household preparations. As there is a huge variety of processed meat products, it is difficult to sort them into categories **(Santarelli et al., 2008)**.

Although consumption of red meat is valuable for the source of protein, iron, vitamin B12, and other vitamins B in the human diet **(Pereira and Vicente, 2013; Salter, 2018)**, however, evidence from epidemiological studies suggests that higher consumption of red meat and processed meat is associated with a higher risk of developing type 2 diabetes mellitus **(Micha, Michas and Mozaffarian, 2012; Pan et al., 2013)** cardiovascular disease (CVD), **(Micha and Mozaffarian**, **2010)** and certain cancers **(Lippi, Mattiuzzi and Cervellin, 2016; Klurfeld, 2015; Wu et al., 2016)**.

Thus, a recent meta-analysis indicates that higher consumption of red meat and processed meat is associated with an increased risk of total, cardiovascular, and cancer mortality **(Wang et al., 2016)**. Several hypotheses have been formulated to explain the potential association of meat intake (mainly processed meat) with the risk of CVD. For instance, the addition of salt or preservatives to meat for conservation purposes may increase the sodium and nitrate content of meat (processed meats could contain about 400% more sodium and 50% more nitrates per g, although this depends strongly on the type of meat and the methods used) **(Linseisen et al., 2006)**.

Nevertheless, findings from randomized controlled trials assessing the effect of red meat intake on CVD risk factors are inconsistent **(Maki et al., 2012; O'Connor et al., 2017)**. Increased cardiovascular risk related to high consumption of red and processed meat has been linked to their high content of saturated fatty acids (SFA) and cholesterol **(Mozaffarian et al., 2010; Rohrmann and Linseisen, 2016; Clonan, Roberts and Holdsworth, 2016)**. Although the recommendation about dietary cholesterol has become obsolete and the role of saturated fatty acids is currently being reconsidered **(Fernandez, 2012; Lawrence, 2013; Mozaffarian and Ludwig, 2015)**, few consumers are up to date regarding these topics.

Scientific hypothesis

This study aims to evaluate the relationship between meat and processed meat consumption frequencies and lipid profile in a group of randomly selected patients with cardiovascular diseases. We assume that a high-frequency consumption of meat and especially processed meat will be associated with higher levels of total cholesterol (T-C), LDL cholesterol (LDL-C), and triglycerides (TG).

MATERIAL AND METHODOLOGY

We evaluated the relationship between meat and meat products consumption frequencies and lipoproteins concentration in a group of randomly selected patients hospitalized in the Cardiocentre Nitra. This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Cardiocentre Nitra and the Ethics Committee of the Specialized Hospital St. Zoerardus Zobor, protocol number 10.6.2014. Patients were 20 – 101 years, (men, the average age was 61.13 ±10.47 years). Selected respondents have either overcome the *myocardial infarction* or were diagnosed with *angina pectoris* and hospitalized after a procedure so-called catheterization. The data necessary for the detection of dietary habits were obtained by a questionnaire method. The questionnaire was applied individually by a single interviewer. The questionnaire was anonymous, its completion was voluntary with only one response to be circled for each question. The questionnaire contained two parts. The first part included questions concerning the socio-demographic situation of the subjects, body height and weight, physical activity, use of tobacco, and to any changes that took place in the participant's. Based on the data on body height and weight, the Body Mass Index (BMI) was calculated for each of the participants by a standard formula. The second part of the questionnaire concerning the analysis of selected dietary habits: number of the consumed meals, their regularity, snacking between the meals, kinds of the consumed snacks, and eating frequency of selected groups of food products. Data collection was carried out simultaneously with a somatometric and biochemical examination of the respondents ensured by the Cardiocentre Nitra. The lipid profile in blood serum was measured by automatic biochemical analyzer BioMajesty® JCA-BM6010/C (DiaSys Diagnostic System GmbH). The following parameters were evaluated: total cholesterol (T-C), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C) and triacylglycerols (TG) because these parameters are considered to be one of the major risk factors for cardiovascular diseases.

Statistical analysis

Data were expressed in figures as mean ± standard deviation (SD) and statistical comparisons between groups were made utilizing one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. Significance was accepted when $p < 0.05$. The program STATISTICA Cz version 10 (TIBCO Software Inc., Palo Alto, California, USA**)** belonging to the available statistical programs and MS Excel 2007 (Microsoft Corporation, Redmond, Washington, USA) was used.

RESULTS AND DISCUSSION

Table 1 and Table 2 describes the basic and demographic statistical characteristics of the study population.

Meat consumption

A well-balanced diet is an important element for health and wellbeing through the whole life span **(Gille, 2010; Löser, 2014)**. It is still widely acknowledged that lean red meat is an important complete protein source, in addition to contributing to essential micronutrient requirements, particularly iron, zinc, and B vitamins **(Biesalski, 2005; McAfee et al., 2010; Webster-Gandy et al., 2012; Pereira and Vicente, 2013)**.

Most participants consumed meat regularly. Only 1 man indicated they were vegetarian. Figure 1 shows the consumption frequency of different kinds of meat as stated by the participants of the study. Table 3 shows the effect of the frequency of consumption of meat on the lipid profile. Pork and poultry were consumed most often: 70.7% (pork) and 63.9% (poultry) of the participants indicated that they consumed these meats 1 – 2 times per week.

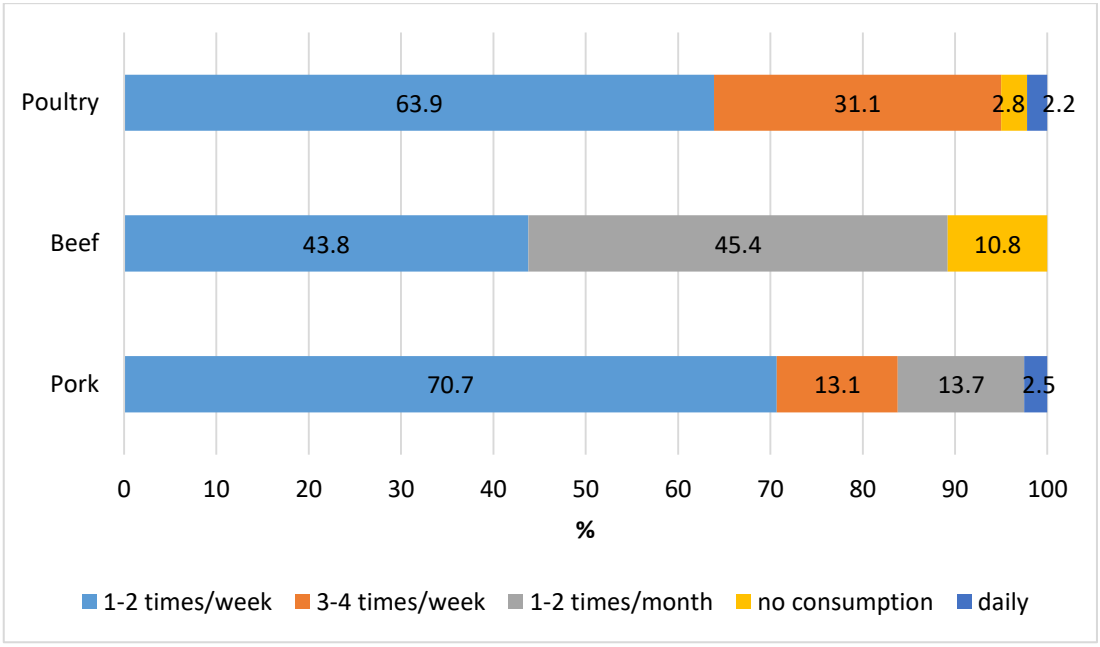

Figure 1 Percentages of consumption frequencies of meat of all respondents (n = 800).

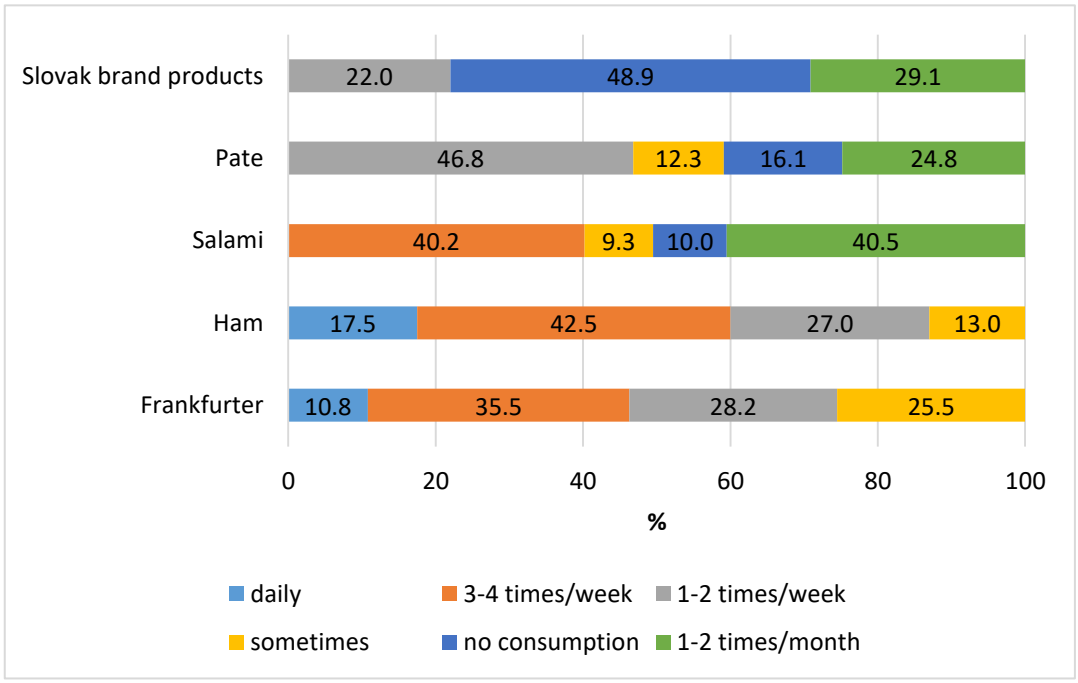

Figure 2 Percentages of consumption frequencies of meat products of all respondents (n = 800).

Table 1 Basic characteristics of study participants (n = 800).

Characteristic	Average ±SD	Min	Max
Age (yrs)	61.13 ±10.47	20.00	101.00
Height (m)	1.75 ±0.07	1.57	1.94
Weight (kg)	90.67 ±15.23	58.00	178.00
BMI ($kg.m^{-2}$)	29.34 ±4.43	17.90	52.00
TC ($mmol.L^{-1}$)	4.68 ±1.25	3.98	9.76
LDL-C ($mmol.L^{-1}$)	2.88 ±0.98	0.61	7.19
HDL-C ($mmol.L^{-1}$)	1.16 ±0.39	0.45	4.01
TG ($mmol.L^{-1}$)	1.73 ±0.93	0.43	7.39
GLU ($mmol.L^{-1}$)	6.66 ±2.31	3.98	24.12

Note: SD – standard deviation; Min – minimum value; Max – maximum value; BMI – Body mass index; TC – total cholesterol; (LDL-C) – LDL cholesterol; (HDL-C) – HDL cholesterol; TG – triglycerides; GLU – glucose.

Table 2 Demographic characteristics of study participants (n = 800).

Characteristics	n	%
Social status		
employed	273	34.2
unemployed	127	15.7
retiree	400	50.1
Family status		
married	582	72.7
divorced	111	13.9
widower	107	13.4
Education		
basic	98	12.2
apprenticeship	234	29.4
secondary	302	37.7
higher	166	20.7
Physical activity		
15 – 30 minutes per day	285	35.6
30 – 60 minutes per day	161	20.1
more than 1 hours per day	354	44.3
Smoker		
yes	251	31.4
no	549	68.6

Table 3 Effect of the frequency of consumption of meat on lipid profil (mmol.L^{-1}).

Frequency of consumption	TC (mmol.L^{-1})	LDL-C (mmol.L^{-1})	HDL-C (mmol.L^{-1})	TG (mmol.L^{-1})
Pork				
3 – 4 times per week	4.87 ±0.16	2.95 ±0.13	1.16 ±0.06	2.03 ±0.12
1 – 2 times per week	4.69 ±0.07	2.81 ±0.06	1.19 ±0.02	1.69 ±0.05
1 – 2 times per month	4.77 ±0.15	2.92 ±0.13	1.18 ±0.06	1.94 ±0.12
p – value	0.032[a]; 0.039[b]	>0.05	>0.05	0.047[a]
Beef				
1 – 2 times per week	4.62 ±0.09	2.84 ±0.39	1.17 ±0.03	1.70 ±0.06
1 – 2 times per month	4.51 ±0.09	3.40 ±0.40	1.21 ±0.03	1.77 ±0.07
no consumption	4.35 ±0.18	3.08 ±0.78	1.17 ±0.06	1.87 ±0.13
p – value	>0.05	>0.05	>0.05	>0.05
Poultry				
daily	4.79 ±0.38	2.90 ±2.32	0.88 ±0.14	1.99 ±0.09
3 – 4 times per week	4.70 ±0.10	2.85 ±0.62	1.18 ±0.04	1.81 ±0.08
1 – 2 times per week	4.55 ±0.07	2.57 ±0.43	1.19 ±0.03	1.74 ±0.05
p – value	>0.05	>0.05	>0.05	>0.05

Note: TC – total cholesterol; (LDL-C) – LDL cholesterol; (HDL-C) – HDL cholesterol; TG – triglycerides; [a]Significant difference between between 1 – 2 times per week and 3 – 4 times per week; [b]Significant difference between between 1 – 2 times per week and 1 – 2 times per month.

Similarly, **Schmid et al. (2017)** found out that, the consumption frequency of beef, pork, and poultry of elderly Swiss population is the highest, with ≥50% of the participants consuming these types of meats at least once a week. **Kopčeková et al. (2015)** in 204 patients with cardiovascular diseases have recorded consumption of meat more than four times per week in 60.28% of men, while more than 30% of men eat meat daily. Respondents indicated poultry and pork as the most commonly consumed meat. **Escriba-Perez et al. (2017)** monitored the consumption of meat in 800 respondents aged 25 to 75 years. Most commonly consumed was chicken, which consumed up to 90.87% of respondents at least once a week. The second most frequently consumed was beef (63.62%), and the third was pork (52.62%).

High concentrations of total cholesterol, LDL cholesterol, and triacylglycerols indicate that the individual is at risk for the onset of cardiovascular disease **(Wooton and Lynne, 2017)**. Elevated serum LDL-C is a long-established risk factor for the development of heart disease; however, the relationship between serum concentrations of LDL-C and dietary cholesterol is not clear **(Zachary et al., 2017)**. Reducing high blood cholesterol is thus important for CVD prevention **(Grundy et al., 1990; Cohen et al., 2006)**. LDL-C is a measure of the total cholesterol content of LDL particles, reflecting both the number of LDL particles and their cholesterol content. Most current guidelines include LDL-C as a primary target for initiating and adjusting lipid-lowering interventions **(Stone et al., 2014; Jacobson et al., 2014)**.

Table 4 Effect of the frequency of consumption of selected processed meat on lipid profile.

Frequency of consumption	TC (mmol.L^{-1})	LDL-C (mmol.L^{-1})	HDL-C (mmol.L^{-1})	TG (mmol.L^{-1})
Frankfurter				
daily	4.45 ±0.18	2.86 ±0.15	1.05 ±0.07	1.52 ±0.14
3 – 4 times per week	4.47 ±0.10	2.75 ±0.08	1.25 ±0.04	1.67 ±0.07
1 – 2 times per week	4.84 ±0.11	2.68 ±0.09	1.11 ±0.04	1.80 ±0.08
Sometimes	4.52 ±0.12	2.62 ±0.10	1.26 ±0.05	1.74 ±0.09
p – value	>0.05	0.046[a]	>0.05	0.012[b]; 0.017[c]
Ham				
Daily	4.50 ±0.14	2.78 ±0.67	1.12 ±0.05	1.62 ±0.11
3 – 4 times per week	4.62 ±0.09	2.76 ±0.43	1.21 ±0.03	1.80 ±0.07
1 – 2 times per week	4.66 ±0.11	2.91 ±0.53	1.14 ±0.04	1.92 ±0.09
Sometimes	4.52 ±0.16	2.84 ±0.45	1.28 ±0.06	1.69 ±0.13
p – value	>0.05	>0.05	>0.05	>0.05
Salami				
3 – 4 times per week	4.55 ±0.12	2.74 ±0.38	1.16 ±0.03	1.87 ±0.06
1 – 2 times per month	4.50 ±0.07	2.70 ±0.28	1.18 ±0.02	1.78 ±0.06
Sometimes	4.88 ±0.16	2.51 ±0.57	1.13 ±0.05	1.49 ±0.11
no consumption	4.53 ±0.15	2.87 ±0.54	1.15 ±0.05	1.99 ±0.12
p – value	>0.05	0.006[d]	>0.05	>0.05
Pate				
1 – 2 times per week	4.62 ±0.08	2.84 ±0.07	1.21 ±0.03	1.78 ±0.07
1 – 2 times per month	4.72 ±0.11	2.83 ±0.09	1.19 ±0.04	1.76 ±0.09
Sometimes	4.76 ±0.16	2.80 ±0.13	1.08 ±0.06	1.70 ±0.13
no consumption	4.43 ±0.14	2.75 ±0.12	1.20 ±0.05	1.65 ±0.11
p – value	>0.05	>0.05	>0.05	>0.05
Slovak brand products (sausage, headcheese and others)				
1 – 2 times per week	4.62 ±0.08	2.83 ±0.07	1.16 ±0.03	1.81 ± 0.07
1 – 2 times per month	4.52 ±0.07	2.81 ±0.06	1.20 ±0.02	1.72 ±0.06
no consumption	4.55 ±0.05	2.77 ±0.04	1.14 ±0.02	1.76 ±0.05
p – value	>0.05	>0.05	>0.05	>0.05

Note: SD – standard deviation; Min – minimum value; Max – maximum value; TC – total cholesterol; (LDL-C) – LDL cholesterol; (HDL-C) – HDL cholesterol; TG – triglycerides; [a]Significant difference between 1 times per week and 2 – 3 times per week; [b]Significant difference between 1 times per week and daily; [c]Significant difference between 2 – 3 times per week and sometimes; [d]Significant difference between 1 – 2 times per week and sometimes.

Red meat intake is commonly considered a risk factor for CVD because of its saturated fat and cholesterol contents **(Rohrmann and Linseisen, 2016)**.

Our results show, that most respondents consume meat 1 – 2 times per week, while we did not notice a significant effect (p >0.05) of the type of meat on the lipid profile.

The highest T-C, LDL-C, and TG values were seen in men who consume pork 3 – 4 times per week. Statistically, significant was the only effect of pork meat on total cholesterol and triglycerides (p <0.05). In the consumption of beef and poultry, there was a non-significant effect on biochemical parameters of blood (p >0.05). **Kopčeková et al. (2015)** in 204 patients with cardiovascular diseases found in both men and women in significantly higher values of HDL for the less frequent consumption, while the values of triglycerides and LDL cholesterol were higher for more frequent consumption. **Kontogianni et al. (2008)** found that a high intake of red meat (more than 8 servings/month) was associated with an increased risk of acute coronary syndrome, but low income (less than four servings/month) showed no association. A recent meta-analysis of 24 randomized controlled trials assessing the effects of red meat intake on CVD risk factors concluded that ≥0.5 serving/day of red meat did not influence blood lipids, lipoproteins, or blood pressure in comparison with <0.5 serving per day **(O'Connor et al., 2017)**. According to **Guasch-Ferré et al. (2019)** findings from the present systematic review and meta-analysis showed that total red meat intake did not differentially influence blood lipids and apolipoproteins, except triglycerides, when all comparison diets were analyzed together.

Processed meat consumption

Processed meat includes meat products that have been modified to change the taste or extend shelf life through curing, smoking, salting, or adding preservatives. Frequently consumed examples are: ham, sausages, salami, bacon, hot dogs, corned beef, beef jerky, ham, canned meat, and meat-based sauces **(Rohrmann et al., 2013; Clonan, Roberts and Holdsworth, 2016)**. Accumulating evidence links excessive consumption of processed meat, and to a lesser extent unprocessed red meat, to an increased risk of obesity, diabetes, cardiovascular diseases, and some cancers **(Micha and Mozaffarian, 2010; Zeng et al., 2019)**.

Figure 2 shows the consumption frequency of different kinds of processed meat as stated by the participants of the study. Table 4 shows the effect of the frequency of

consumption of selected processed meat on the lipid profile.

Most respondents consume frankfurters (hot dogs) 3 – 4 times per week (35.5%) or 1 – 2 times per week (28.2%). Approximately 11% of respondents consume frankfurters daily, which is associated with non-significant higher LDL and lower HDL levels (p >0.05). We recorded a significant effect (p <0.05) of the consumption of frankfurters on the lipid profile. Almost half of the respondents consume meat pate 1 – 2 times per week and 24.8% only 1 – 2 times per month. Most respondents consume ham 3 – 4 times per week (42.5%), daily consumption of ham was recorded in 17.5% of patients. Different frequency of ham and meat pate consumption show non-significant changes for the lipid profile (p >0.05). Up to 40.2% of respondents consume salami 3 – 4 times/week, and we recorded a significant effect on LDL levels between consumption 1 – 2 times/week and sometimes (p <0.05). We also monitored the consumption of traditional Slovak brand products (sausage, headcheese, and others). Processed meats such as sausages have a higher content of saturated fatty acids and cholesterol than fresh red meat; reaching the proportion of fat in sausages more than 50% of weight **(Lajous et al., 2014)**. Almost half of the respondents (48.9%) state that they do not consume sausage, headcheese, and others at all. 29.1% of patients consume this product 1 – 2 times per month and 22.0% 1 – 2 times per week. Respondents who consume sausage, headcheese, and other products 1 – 2 times a week have non-significant higher T-C, LDL, TG, and lower HDL compared to less frequent consumption.

Micha and Mozaffarian (2010) in their meta-analysis of 20 studies (17 prospective cohorts and 3 cases control studies) that included 1218380 individuals concluded that intake of processed, but not red, meat was associated with an increased incidence of coronary heart disease and the authors speculate that the higher sodium and nitrate content of processed meat might contribute to their impact on CVD. This investigation in EPIC, including nearly half a million participants across 10 European countries and more than 5000 cardiovascular events, confirms that consumption of processed meat is strongly associated with CVD risk, and that consumption of unprocessed red meat has little to no association **(Rohrmann et al., 2013)**. **Watson and Preedy (2013)** report the results of research that focused on the effect of regular consumption of processed red meat (25, 75 grams or more per day) in 37035 healthy people. Probands who consumed 75 or more grams of meat products per day were 28% more likely to have heart failure than those who consumed less than 25 grams of meat products per day. Probands who consumed the most meat products had at least twice the risk of dying from CVD compared to those who consumed them.

CONCLUSION

In our study, we observed the associations between consumption of meat and meat products and lipid profile in a group of randomly selected patients with cardiovascular disease. Most participants consumed meat and meat products regularly. High consumption of meat, mainly pork and processed meat seems to be associated with higher levels of total cholesterol, LDL cholesterol, and triglycerides. This study offers support to the perception that food consumption is an important determinant in cardiovascular disease and its risk factors. As diet is a modifiable CVD risk factor, health promotion activities should consider specific advice on lowering processed meat consumption and, to a lesser extent, red meat consumption. Results like ours strongly suggest that to accurately investigate this relationship in the future, both red and processed meat must be analyzed separately. Further studies are needed to examine the role of meat and meat products in the prevention and management of cardiovascular diseases. A healthy lifestyle, including a healthy diet is the best strategy for the prevention of cardiovascular disease and other diseases.

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DIETARY SUPPLEMENTATION WITH MAGNESIUM CITRATE MAY IMPROVE PANCREATIC METABOLIC INDICES IN AN ALLOXAN-INDUCED DIABETES RAT MODEL

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ABSTRACT

The purpose of the current study was to evaluate the protective properties of dietary magnesium supplementation on pancreatic tissue of rats with alloxan-induced *diabetes mellitus*. Twenty-five male Wistar rats were split into five groups (control, diabetes, diabetes with 100 mg Mg daily, diabetes with 250 mg Mg daily, diabetes with 500 mg Mg daily) with feeding supplementation starting on day 1, diabetes induction on day 21, and animal sacrifice on day 30. Fasting glucose in blood serum was measured on days 21, 25, 27, and day 30. Glucose metabolism enzymes, namely, lactate dehydrogenase and glucose-6-phosphate dehydrogenase, were measured in pancreatic tissue upon the sacrifice, as well as lipid peroxidation, antioxidant system protective enzymes (catalase and superoxide dismutase), and glutathione system components (glutathione reductase, glutathione peroxidase, and glutathione reduced). Pearson correlation coefficients showed strong negative correlation between serum glucose (control and diabetic animals) and glucose metabolism enzymes, catalase, superoxide dismutase, glutathione peroxidase in pancreatic tissue ($r > -0.9$, $p < 0.05$), moderate negative correlation with reduced glutathione ($r = -0.79$, $p < 0.05$), moderate positive correlation with lipid peroxidation index ($r = +0.67$, $p < 0.05$), weak correlation with glutathione reductase ($r = -0.57$, $p < 0.05$). Magnesium supplementation slowed down diabetes onset considering fasting glucose levels in rats ($p < 0.05$), as well as partially restored investigated dehydrogenase levels in the pancreas of rats comparing to diabetes group ($p < 0.05$). The lipid peroxidation index varied between treatments showing the dose-dependent influence of Mg^{2+}. Magnesium supplementation partially restored catalase and superoxide dismutase activities in pancreatic tissue, as well as glutathione peroxidase and reduced glutathione levels ($p < 0.05$), while glutathione reductase levels remained unaffected ($p > 0.05$). The obtained results suggested a model, where magnesium ions may have a possible protective effect on pancreatic tissue against the negative influence of alloxan inside β cells of the pancreas.

Keywords: alloxan; diabetes; rat model; magnesium oral supplementation; pancreas

INTRODUCTION

Diabetes mellitus is a major health-related problem worldwide, with the number of people with diabetes increasing from 108 million in 1980 to 422 million in 2014, according to the WHO **(WHO, 2018)**. Dietary habits, together with a genetic predisposition, may influence *diabetes mellitus* development in healthy individuals. Magnesium is a macroelement involved in virtually all biochemical pathways in cells **(de Baaij, Hoenderop and Bindels, 2015)**. Considering its importance, the serum Mg^{2+} levels are maintained between 0.7 and 1.05 mM level in blood serum **(de Baaij, Hoenderop and Bindels, 2015)**. Low serum Mg^{2+} levels could be attributed to either dietary restriction, impaired intestinal absorption, or increased renal wasting. Hypomagnesemia has been long reported as a risk factor for type 2 *diabetes mellitus* development, as a contributing reason for **(Dong et al., 2011)**, as well as a consequence of insulin resistance **(Pham et al., 2007; Chaudhary, Sharma and Bansal, 2010)**. "A vicious cycle" of hypomagnesemia described by **Gommers et al. (2016)** implies the necessity of Mg^{2+} for insulin receptor autophosphorylation and therefore preventing insulin resistance from one side, while circulating excess of insulin being able to activate renal Mg^{2+} channel transient receptor potential melastatin type 6, which stimulates the secretion of Mg^{2+} with urine. Magnesium influences glucose metabolism and insulin action in several ways. Firstly, it is involved in the autophosphorylation of β-subunits of insulin receptor through tyrosine kinase activity binding to ATP. Rats with hypomagnesemia had reduced levels of the phosphorylated insulin receptor, mimicking insulin resistance **(Paxton and Ye, 2005; Suárez et al., 1995)**. Secondly, recent studies showed that

oral Mg^{2+} supplementation increased GLUT1 and GLUT4 expression in muscles of STZ-induced diabetic rats and mice, resulting in lowering serum glucose levels and its uptake by muscle tissue **(Biddinger and Kahn, 2006; Solaimani et al., 2014)**. Thirdly, Mg^{2+} is an important anti-inflammatory mediator, and hypomagnesemia increased production of proinflammatory interleukin 1, tumor necrosis factor-α by adipocytes, causing the production of reactive oxygen species that may lead to chronic inflammation, insulin resistance, and reduced GLUT4 activity **(Rodriguez-Moran and Guerrero-Romero, 2004; Weglicki et al., 1992)**. And finally, Mg^{2+} affects insulin production in pancreatic β cells. Under normal physiological conditions, increased blood glucose levels stimulate the influx of glucose into β-cell via GLUT2 transporter, followed by its conversion to glucose-6-phosphate (G6P) by glucokinase, which is a " glucose sensor" for insulin secretion. G6P is further metabolized through glycolysis and Krebs cycle, producing ATP, excess of which directly induces closure of K_{ATP} channel Kir6.2. Closure of this channel causes depolarization of plasma membrane, the opening of a voltage-dependent influx of Ca^{2+} ions, which causes exocytosis of insulin into the bloodstream. From one side, Mg^{2+} directly positively influences glucokinase activity by acting as a cofactor of adenine nucleotides, though it may be happening even at sub-physiological levels. Conversely, Mg^{2+} initiates K_{ATP} channel opening through MgATP, negatively affecting L-type Ca^{2+} channels, an influx of Ca^{2+} into β cells, and restraining insulin release. However, those are short-time negative consequences, while in a long run Mg^{2+} may improve β cells functionality, similar to cardiomyocytes, where low Mg^{2+} level was shown to decrease expression of L-type Ca^{2+} channels **(Gommers et al., 2016)**. It was shown that in humans without diabetes decreased serum Mg^{2+} was associated with decreased insulin secretion **(Rodríguez-Morán and Guerrero-Romero, 2011)**. Similarly, supplementation of individuals without diabetes with $MgCl_2$ significantly improved β-cell function and insulin secretion **(Guerrero-Romero and Rodríguez-Morán, 2011)**.

Alloxan is a cytotoxic glucose analog, which selectively binds to GLUT2 receptors of pancreatic β cells and enters them, causing necrotic cell death, thus modeling type 1 diabetes **(Lenzen, 2008)**. Alloxan is highly unstable in solutions with neutral pH and bloodstream and decomposes to alloxanic acid within minutes **(Szkudelski, 2001)**. A single intraperitoneal injection of 150 $mg.kg^{-1}$ of live weight is sufficient to cause alloxan-induced diabetes in fasting rats, causing permanent hyperglycemia between 24 to 48 hours post-injection **(Ighodaro, Adeosun and Akinloye, 2017)**. Being a thiol reagent, alloxan inhibits glucokinase ("glucose sensor"), and, therefore, glucose phosphorylation in β cells, initially causing rapid ATP increase followed by rapid insulin secretion and severe hypoglycemia, followed by halting and inhibition of insulin secretion **(Szkudelski, 2001; Lenzen, 2008; Ighodaro, Adeosun and Akinloye, 2017)**. More importantly, alloxan enters redox cyclic reaction coupled with its oxidized form, dialuronic acid, which spontaneously autooxidizes to generate toxic superoxide radicals and hydrogen peroxide, commonly known as reactive oxygen species (ROS) **(Munday, 1988; Winterbourn and Munday, 1989; Lenzen, 2008)**. Thiol groups, specifically, but not limited, to reduced glutathione are required for dialuronic acid production **(Szkudelski, 2001)**. A temporary compound of alloxan-glutathione, namely, "compound 305", is formed during each cycle, resulting in decreased GSH/GSSG ratio and depletion of reduced glutathione **(Munday, 1988; Szkudelski, 2001)**. The ultimate damage is caused by another ROS, hydroxyl radicals, which acts together with superoxide radicals and hydrogen peroxide, causing massive cellular damage, and ultimately apoptosis. Interestingly, few reviews noted massive Ca^{2+} influx in β cells and subsequent disruption of intracellular calcium homeostasis as one of the major reasons for β cell necrosis as well **(Szkudelski, 2001; Ankur and Shahjad, 2012)**. Antioxidant enzymes, namely, superoxide dismutase (transforming superoxide radical into hydrogen peroxide), and catalase (transforming hydrogen peroxide into water and oxygen), protect cells against ROS produced by alloxan **(Grankvist et al., 1979; Winterbourn and Munday, 1989)**. Glutathione peroxidase also detoxifies cells from hydrogen peroxide, though it requires reduced glutathione as a substrate. Cell debris, produced by necrotic dying β cells, are quickly scavenged by macrophages.

The purpose of the current study was to evaluate the influence of magnesium dietary supplementation on the duration and late progression of alloxan-induced diabetes related to pancreatic metabolic indices, including enzymes of glucose metabolism (lactate dehydrogenase, LDH, and glucose-6-phosphate dehydrogenase, G6PD), state of lipid oxidation (lipid hydroperoxide, LOOH), antioxidant enzymes (catalase, CAT, and superoxide dismutase, SOD), and glutathione system (glutathione peroxidase, G-Per, glutathione reductase, G-Red, and reduced glutathione, GSH). To the best of our knowledge, this study relative to pancreatic health state after late progression of alloxan-induced diabetes, with or without oral magnesium supplementation, was not performed yet. Additionally, we measured fasting serum glucose levels in rats with alloxan-induced diabetes, with or without dietary magnesium supplementation, as a time-dependent variable influenced by diabetes onset and progression.

Scientific hypothesis

We hypothesize that magnesium supplementation will not prevent, but may reduce the toxic effects of alloxan on pancreatic β cells. This may include the restoration of pancreatic glucose metabolism enzyme function back to the levels of the healthy control rats; decrease in LOOH comparing to alloxan-treated rats, reducing their levels back to the levels found in healthy control rats; activation of CAT and SOD, as protectors against ROS; increase in levels of G-SH and glutathione system enzymes, namely, G-Red and G-Per. Additionally, we hypothesize that magnesium supplementation may reduce fasting blood glucose levels in alloxan-induced diabetic rats due to the improved pancreatic function.

MATERIALS AND METHODOLOGY

The alloxan-induced diabetes rat model

Twenty-five white Wistar rats (ca. 140 g each, same age) were used for the experiments. The animals were kept in cages under standard conditions (20 – 25 °C, 40 – 45% air relative humidity) with free access to compound feed, while water was strictly supplemented (20 mL daily). Compound feed composition remained unknown. All procedures with animals were carried out according to the "European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes" (Strasbourg, France, Council of Europe, March 18, 1986) and were approved by the local institutional ethical committee.

The rats were divided into five groups, 5 rats per group: intact control (group 1, C); induced diabetes (group 2, D); magnesium supplementation (100 mg Mg^{2+} kg^{1} weight, daily dose) with diabetes (group 3, Mg100-D); magnesium (250 $mg.kg^{-1}$) with diabetes (group 4, Mg250-D); magnesium (500 $mg.kg^{-1}$) with diabetes (group 5, Mg500-D). Day 0 was the date of dietary supplementation start, while alloxan induction of diabetes was performed on day 21, and all animals were sacrificed on day 30. Magnesium citrate ($C_6H_6O_7Mg$) was dissolved in tap water in the amount of 6.17, 15.44, and 30.88 mg per mL corresponding to 14, 35, or 70 mg Mg^{2+} in 20 mL water per animal, which relates to 100, 250, and 500 mg Mg^{2+} per kg of live weight. A single intraperitoneal injection of alloxan monohydrate ("Sinbias", Ukraine) in 0.85% physiological saline was performed on each rat from groups 2, 3, 4, and 5 (150 $mg.kg^{-1}$ of body weight) after 24-hours fasting on the 21st day from the start of the experiment. All animals were sacrificed under ether anesthesia by decapitation on day 30, followed by removal of the pancreas within 2 minutes after the death. The removed pancreas was kept on ice for 5 to 6 minutes followed by bleeding through repeated perfusion with physiological saline (0.85% NaCl, 4 °C). The pancreas was chopped coarsely by scissors. One gram of chopped tissue was homogenized with 9.0 mL of 5 mM Tris-HCl buffer (1:10 dilution), pH 7.4, for 50 seconds at 0 °C (MRTU-421505-63, Ukraine). The homogenate was filtered through two layers of cheesecloth into a centrifuge tube followed by centrifugation (3,000 g, 10 minutes, 0 °C). The supernatant was used immediately for biochemical markers quantification and called "tissue sample" throughout the research paper.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Fasting blood serum glucose determination

Glucose determination was done on venous blood drawn from rat tails using glucose oxidase method (Gamma TM mini glucometer) after at least 6-hours fasting on days 21, 23, 25, 27, and day 30 (sacrifice day). The principle of the method is based on the reaction catalyzed by glucose oxidase:

$$\text{Glucose} + O_2 \rightarrow \text{Glucolactone} + H_2O_2 \quad (1)$$

using test strips and calibration strips supplied by the manufacturer.

Lipid hydroperoxide (LOOH) determination

LOOH determination is based on the interaction of ethanol extracts of lipid hydroperoxides with ammonium thiocyanate after precipitation of proteins with trichloroacetic acid (TCA). The original method developed by **Wagner, Clever and Peters (1947)**, and later updated and modified by **Mihaljević, Katušin-Ražem and Ražem (1996)**, was adapted. Briefly, 0.2 mL of tissue sample was placed in a centrifuge tube with 2.8 mL of ethanol (95%) and 0.05 mL of 50% TCA at 4 °C. The tube was tightly closed and shaken for 5 minutes, followed by centrifugation, resulting in protein precipitation (3,000 g, 10 minutes, 4 °C). 1.5 mL of the resulting supernatant was mixed with 1.2 mL of ethanol, 0.02 mL of concentrated HCl, and 0.03 mL of 1% Mohr's salt, $Fe(NH_4)_2(SO_4)_2$, in 3% HCl. The mixture was shaken, followed by addition of 0.2 mL of 20% NH_4SCN. Optical absorbance (A_{480}) was measured after 10 minutes of incubation time (spectrophotometer UNICO S1205, λ = 480 nm). The control sample was prepared by tissue sample replacement with 0.2 mL of deionized water. LOOH in samples was calculated using the following equation:

$$\Delta A_{480}\ \text{LOOH}.g^{-1} = DF \times (A_{480\ exp} - A_{480control}) \quad (2)$$

The obtained results were expressed as the difference in optical absorbance between experimental and control samples (λ = 480 nm), adjusted to 1 g of pancreatic tissue (ΔA480 $LOOH.g^{-1}$) by dilution factor DF.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Total protein determination

Protein concentration in the tissue sample was determined by the Lowry method using standardized kits (SIMCO Ltd, Ukraine) according to the manufacturer's instructions. The method is based on the formation of non-ferrous products of the interaction of aromatic amino acids with the Folin-Ciocalteu reagent in combination with the biuret reaction to peptide bonds of the protein **(Lowry et al., 1951)**. The results were expressed in mg of protein per mL of the tissue sample using spectrophotometer UNICO S1205 for absorbance measurements. The protein concentration of tissue samples from the pancreas of each rat was used to estimate enzyme activity per mg of protein in the subsequent analysis of SOD, CAT, G-Per, G-Red, G6PD, and LDH enzymatic activities in tissue samples.

Superoxide dismutase (SOD) determination

The principle of the method for superoxide dismutase (EC 1.15.1.1) activity determination is based on the ability of the enzyme to compete with nitroblue tetrazolium for the superoxide anion radicals, which are formed as a result of aerobic interaction of NADH and phenazine methosulfate **(Ponti et al., 1978)**. As a result of this reaction, nitroblue tetrazolium is reduced to formazan dye. In the presence of SOD, the percentage of nitroblue tetrazolium reduction is reduced. Enzyme activity was determined by the percentage of blockage of formazan dye formation **(Durak et al., 1993)**. Briefly, 1.5 mL of incubation mixture (containing 37 mg EDTA-Na_2, 330 mg nitrotetrazolium blue, 55 mg phenazine methosulfate, and

0.3 mL 0.15 M phosphate buffer, pH 7.8) was mixed with 0.1 mL of a tissue sample and 0.1 mL of 1 mM NADH in Tris-EDTA buffer (pH 8.0) followed by 10 minutes incubation at room temperature. Optical absorbance was measured against water (UNICO S1205, $\lambda = 540$ nm). The control sample was prepared using distilled instead of a tissue sample. The enzyme activity was determined with the following equation:

$$\% \text{ blockage} = 100 \times (A_c - A_t)/A_c \quad (3)$$

where A_c – optical absorbance of the control sample; A_t – optical absorbance of the test sample. Superoxide dismutase activity was expressed in relative units per 1 mg of protein (1 relative unit = 50% blocking).

Catalase (CAT) determination

The principle of the method for catalase (EC 1.11.1.6) activity determination is based on the ability of H_2O_2 to form a stable colored complex with molybdenum salts. According to **Góth (1991)**, the color intensity of molybdenum peroxide compounds depends on the amount of H_2O_2 in solution; and therefore, on the activity of catalase in the sample. The catalase reaction was started by mixing 0.1 mL of a tissue sample with 1 mL of 0.05 M Tris-HCl buffer (pH 7.8) and 2 mL of 0.03% H_2O_2. Blank included 1 mL of 4% $(NH_4)_2MoO_4$ in 0.025 N H_2SO_4 and 2.0 mL H_2O_2. The reaction in the test sample was stopped after 10 minutes by adding 1 mL of 0.25 N H_2SO_4 and 1 mL of 4% $(NH_4)_2MoO_4$. To complete the blank, 1 mL of 0.25 N H_2SO_4 and 0.1 mL of tissue sample was added to the blank.

Samples were centrifuged for 5 minutes at 3,000 g. Optical absorbance was determined spectrophotometrically (UNICO S1205, $\lambda = 410$ nm). The catalase activity in $\mu mol.min^{-1}.mg^{-1}$ was calculated using the following equation:

$$E_a = (\Delta A \times DF)/(\varepsilon \times t \times C \times l) \quad (4)$$

Where: ΔA – difference between the optical absorbance of blank and test samples; DF – dilution factor of the original tissue sample; ε – molar extinction coefficient of the H_2O_2 complex with ammonium molybdate at $\lambda = 410$ nm, equal to 22200 $M^{-1}.cm^{-1}$; C – tissue sample protein concentration, $mg.mL^{-1}$; t – reaction time, 10 minutes, l – optical path, 1 cm.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Glutathione peroxidase (G-Per) determination

The activity of glutathione peroxidase (EC 1.11.1.9) was measured through the oxidation rate of reduced glutathione in the presence of tertiary butyl hydroperoxide **(Moin, 1996)**. Change in absorbance is due to the interaction between SH-groups and Ellman's reagent (0.01 M solution of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in methanol) with the formation of a colored product, 5'-thio-2-nitrobenzoic acid. The amount of the latter is directly proportional to the number of -SH groups that reacted with Ellman's reagent. Briefly, 0.83 mL of 4.8 mM reduced glutathione in 0.1 M Tris-HCl buffer (pH 8.5), containing 6 mM EDTA and 12 mM NaN_3, was added to 0.2 mL of the tissue sample. The mixture was incubated at 37 °C for 10 minutes. Following incubation, 0.07 mL of 20 mM tertiary butyl hydroperoxide was added and the mixture was incubated for an additional 5 minutes. The reaction was stopped by adding 0.2 mL of 20% trichloroacetic acid at 4 °C. A blank sample was prepared identically, but with distilled water instead of the tissue sample. The samples were centrifuged for 10 minutes at 3000 g. The supernatant (0.02 mL) was mixed with 2 mL of 0.1 M Tris-HCl buffer (pH 8.5) and 0.02 mL of Elman's reagent. After 5 minutes incubation, the samples were analyzed spectrophotometrically (UNICO S1205, $\lambda = 412$ nm). The G-Per result was expressed in $\mu mol.min^{-1}.mg^{-1}$ protein and calculated using the following equation:

$$E_a = (\Delta A \times DF \times C_s)/(A_s \times t \times C) \quad (5)$$

Where: ΔA – the difference between optical absorbance of the control and experimental samples; DF – dilution factor of the original tissue sample; A_s – absorbance of the standard G-SH solution; C_s – concentration of the standard G-SH solution; C – protein concentration in the tissue sample, $mg.mL^{-1}$; t – reaction time, 5 minutes.

Glutathione reductase (G-Red) determination

The activity of glutathione reductase (EC 1.8.1.7) was determined spectrophotometrically as the rate of glutathione reduction in the presence of NADPH as suggested by **Carlberg and Mannervik (1985)**. The reaction mixture contained 2 mL of 0.15 M phosphate buffer, pH 7.4; 0.2 mL of 10 mM EDTA**;** 0.5 mL of 7.5 mM oxidized glutathione, 0.1 mL of 2 mM NADPH; and 0.2 mL of the test sample. The enzyme activity was determined by measuring the reduction in NADPH content within 10 minutes at 37 °C (UNICO S1205, $\lambda = 340$ nm). The activity of G-Red was expressed in $\mu mol(NADPH).min^{-1}.mg^{-1}$ protein and calculated using equation 4, where ΔA – absorbance difference between the start and end time of the reaction; DF – dilution factor of the tissue sample; ε – molar extinction coefficient for NADPH, $\varepsilon = 6220\ M^{-1}.cm^{-1}$; t – reaction time, 10 minutes.

Reduced glutathione (G-SH) determination

The content of reduced glutathione (G-SH) was determined by the level of 5'-thio-2-nitrobenzoic acid formation as a result of the interaction of the glutathione – SH groups with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at $\lambda = 412$ nm as per method developed by **Beutler, Duron and Kelly (1963)**. The following reagents were used: a precipitating reagent (glacial metaphosphoric acid – 6.68 g; EDTA – 0.80 g; sodium chloride – 120.0 g; distilled water to 400 mL); 0.3 M Na_2HPO_4 solution in distilled water; Elman's reagent (0.04% solution of 5.5-dithiobis-2-nitrobenzoic acid in 1% solution of trisodium citrate) **(Rahman, Kode and Biswas, 2006)**.

Stage I. Sample: tissue sample – 2 mL, precipitating reagent – 3 mL; control: distilled water – 2 mL, precipitating reagent – 3 mL. The mixtures were kept at room temperature for 5 minutes followed by centrifugation

at 3,500g, through which protein-free supernatant was obtained.

Stage II. Sample: supernatant from stage I – 2 mL, 0.3 M Na_2HPO_4 – 8 mL, Elman's reagent – 0.1 mL; Control: control supernatant from stage I – 2 mL; 0.3 M Na_2HPO_4 – 8 mL, Elman reagent– 0.1 mL. After 5 minutes of incubation, the absorbance measurement of the sample against control was performed (UNICO S1205, $\lambda = 412$ nm). The calculation of the amount of G-SH in the pancreas in mmoles per gram of tissue was estimated against G-SH standard solution with known concentration C_s using equation 6:

$$N_{GSH} = (\Delta A_{412} \text{ x } DF \text{ x } C_s)/A_{412s} \quad (6)$$

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Lactate dehydrogenase (LDH) and glucose-6P-dehydrogenase (G6PD) determination

LDH and G6PD enzymatic activity were determined using spectrophotometric methods based on the coupled oxidation/reduction systems of nicotinamide coenzymes (UNICO S1205, 37 °C, NADH and NADPH molar extinction coefficient $\varepsilon = 6220$ at $\lambda = 340$ nm, t = 5 minutes of enzymatic reaction). The reaction was carried in 0.2 M tris-HCl buffer (pH 7.5) with a total volume of 3 mL, including 0.2 mL of the tissue sample. The final concentrations of the reaction components were as follows:

LDH – 1×10^{-3} M sodium pyruvate, 5×10^{-5} NADH, 3×10^{-3} M $MgCl_2$ **(Sevela and Tovarek, 1959)**.

G6PD – 1×10^{-3} M glucose-6-phosphate, 5×10^{-3} M $MgCl_2$; 0.5×10^{-4} M $NADP^+$ **(Evans, 2009)**.

The enzymatic activity was expressed in $nmole.min^{-1}.mg^1$ protein and calculated using equation 4.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Statistical analysis

Two-factorial ANOVA was used to analyze the influence of treatment (D, Mg100-D, Mg250-D, Mg500-D) and time of experiment onset (day 21, day 25, day 27, and day 30), as well as their interaction (treatment x time), on the fasting glucose levels in rats. If the influence of factors or their interaction was significant ($p < 0.05$), means were separated using the Fisher LSD procedure.

Pearson correlation coefficients were calculated between paired serum glucose levels of the control (C) and diabetes-induced rats (D) on day 30 and LOOH, SOD, CAT, G-Per, G-Red, G-SH, G6PD, and LDH levels in pancreas after animal sacrifice.

One-way ANOVA was used to analyze the influence of treatment (C, D, Mg100-D, Mg250-D, Mg500-D) on carbs metabolism enzyme activity, namely, G6PD and LDH; on lipid oxidative damage index, namely, LOOH level; on antioxidant enzymes activity, namely, SOD and CAT; and on glutathione system, namely, G-Per and G-Red activities and G-SH levels, in pancreatic tissue. If the influence of the factor was significant ($p < 0.05$), means were separated using the Fisher LSD procedure.

RESULTS AND DISCUSSION

As expected, oral magnesium supplementation did not prevent alloxan induction of diabetes, though somewhat significantly slowed its progression (Figure 1, $p < 0.05$). There was a significant influence of both factors, treatment (D, Mg100-D, Mg250-D, Mg500-D) and time of experiment onset (day 21, day 25, day 27, and day 30), on fasting serum glucose concentration ($p < 0.05$, Figure 1), though their interaction (treatment x time) did not affect ($p > 0.05$). Glucose levels increased gradually with the time of the experiment onset (Figure 3), while 250 $mg.kg^{-1}$ magnesium-dose had the most profound effect on the fasting glucose reduction (Figure 2). Similarly, **Abayomi et al. (2011)** showed that a single intraperitoneal injection of magnesium (100 and 150 $mg.kg^{-1}$ live weight) one hour before alloxan-induced diabetes (120 $mg.kg^{-1}$ live weight) in rats improved fasting glucose levels on days 2, 5, 7, and 10 compared to diabetes alone, but still was significantly higher comparing to control. Additionally, **Ige et al. (2010)** showed a similar effect of a single intraperitoneal injection of magnesium (100 $mg.kg^{-1}$) one hour before diabetes induction in rats even at a higher level of alloxan dose (150 $mg.kg^{-1}$ live weight).

The Pearson correlation coefficients between serum glucose levels (C and D groups) and investigated indices are shown in Table 1. The increased glucose levels, as a result of alloxan-induced diabetes, had strong negative correlations with LDH and G6PD enzymes, possibly indicating cellular damage and reduced metabolic activity of pancreatic cells; strong negative correlations with CAT, SOD, G-Per, possible indicating damage of antioxidative enzyme system as a result of confronting ROS produced by alloxan; medium negative correlations with G-SH, indicating G-SH consumption and possible impairment during alloxan redox cycling with dialuronic acid. A weak negative correlation was observed with G-Red, and moderate positive correlation with LOOH, indicating residual oxidative damage to the lipids of the cell membranes. These weak positive correlations could be because alloxan-induced diabetes was already established and necrotic β cells already removed by macrophages before the day of animal sacrifice (day 30).

Polyunsaturated fatty acids (PUFAs), which in their esterified state are present in membrane or storage lipids, are prone to ROS-induced peroxidation, resulting in the damage to the biomembranes. Final products of lipid peroxidation (LOOH) are reactive aldehydes, which are relatively stable, can diffuse far from the location of oxidative injury, and may act as second messengers or free radicals **(Gasparovic et al., 2013)**. Levels of LOOH were significantly different between treatments as shown in Table 2 ($p < 0.05$). LOOH increased non-significantly in the diabetes group, and was significantly decreased compared to diabetes in Mg250-D group ($p < 0.05$). Paradoxically, the highest LOOH levels were observed at high Mg^{2+} supplementation (500 $mg.kg^{-1}$), possibly showing that at such levels influence of Mg^{2+} may be negative, which is also confirmed by increased fasting serum glucose in this group (Figure 2). Though the major effect of targeted alloxan cytotoxic action on β cells is massive production of ROS, such as superoxide radical, hydrogen peroxide, and hydroxyl radical **(Munday, 1988; Winterbourn and Munday, 1989)**, the result is apoptosis

of β cells followed by cell debris removal. The determination of LOOH was done long after alloxan major damage and after its degradation in rat body (day 10 after the onset of diabetes), and presumably all damaged cells were either removed or repaired back to normal. Therefore, LOOH values could be non-informative.

Another possibility is the lack of the precision in determination of these values due to the low number of observations, causing an absence of significant differences between treatments.

Evaluation of enzyme activity, due to their protein nature, and sensitivity to various processing conditions, as well as pathological processes, is widely used in industry, life science, and medical research **(Tokarskyy et al., 2009; Yaremchuk and Posokhova, 2011; Lykhatskyi et al., 2019)**.

Table 1 Pearson correlation coefficients (p <0.05) between fasting serum glucose levels in control (C) and diabetic (D) groups vs. investigated indices (LOOH, SOD, CAT, G-Per, G-Red, G-SH, LDH, G6PD).

	LOOH	SOD	CAT	G-per	G-red	G-SH	LDH	G6PD
Pearson correlation coefficient, r	+0.67	-0.92	-0.98	-0.91	-0.57	-0.79	-0.93	-0.95

Table 2 Influence of treatment (control, diabetes, diabetes with 100, 250, and 500 mg Mg supplementation) on LOOH levels in pancreatic tissue of rats upon sacrifice on day 30, with standard deviation included.

	C ±SD	D ±SD	Mg100-D ±SD	Mg250-D ±SD	Mg500-D ±SD
LOOH, $\Delta A_{480}.g^{-1}$	0.133 ±0.014 bd*	0.165 ±0.021 b	0.143 ±0.014 bc	0.133 ±0.013 cd	0.210 ±0.011 a

Note: *Means with the same letters are not significantly different (p >0.05).

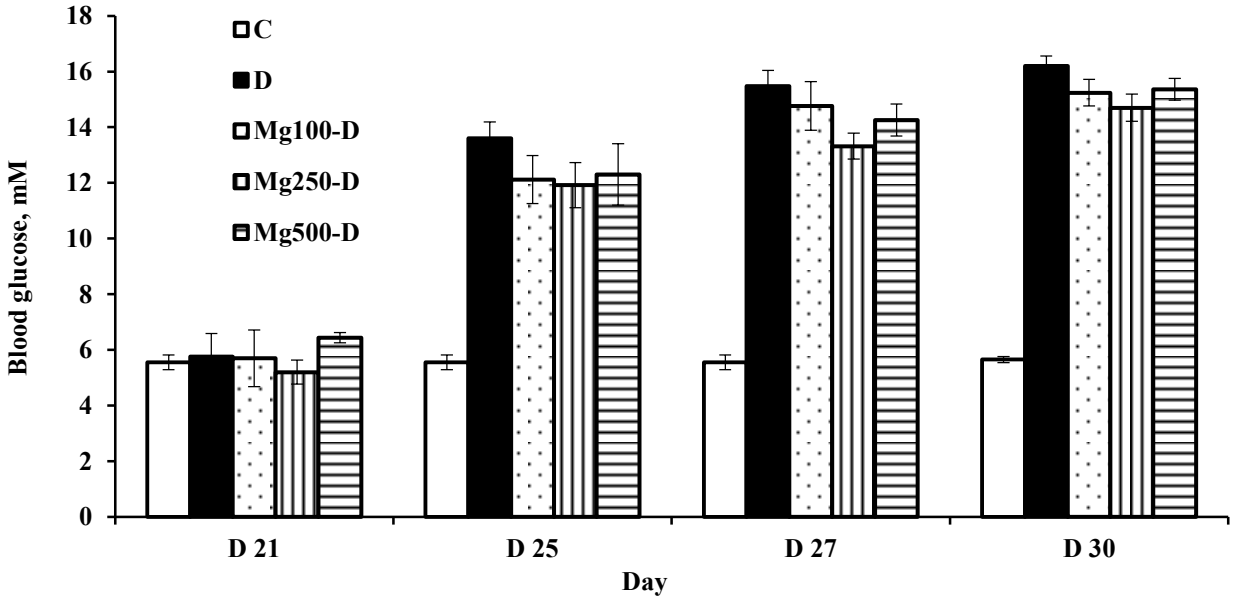

Figure 1 Mean values of fasting blood serum glucose levels in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on days 21, 25, 27, and sacrifice day (day 30). Control (C) is shown for reference. Error bars reflect standard deviations. Note: Means with the same letters are not significantly different (p >0.05).

Table 3. Influences of treatment (control, diabetes, diabetes with 100, 250, and 500 mg Mg supplementation) on G-Per (p <0.05), G-Red (p =0.7), and G-SH (p <0.05) levels in pancreatic tissue of rats upon sacrifice on day 30, with standard deviation included.

	C ±SD	D ±SD	Mg100-D ±SD	Mg250-D ±SD	Mg500-D ±SD
G-Per, $\mu mol(G\text{-}SH).min^{-1}.mg^{-1}$	3.30 ±0.43 ab*	1.80 ±0.14 c	2.84 ±0.31 b	3.97 ±0.42 a	2.44 ±0.28 bc
G-Red, $\mu mol(NADPH).min^{-1}.mg^{-1}$	0.028 ±0.001 a*	0.024 ±0.002 a	0.025 ±0.002 a	0.026 ±0.005 a	0.024 ±0.004 a
G-SH, $mmol.g^{-1}$	0.031 ±0.008 a*	0.015 ±0.005 b	0.024 ±0.004 a	0.028 ±0.004 a	0.023 ±0.004 ab

Note: * Same letters within the same lane mean non-significant difference (p >0.05).

Results of CAT and SOD determination are shown in Figure 4 and Figure 5, respectively. Treatment had a significant influence on both enzymes (p <0.05). Alloxan-induced diabetes caused a significant decrease in activities of both enzymes, while magnesium supplementation restored those values, causing even over-production of catalase. Both enzymes are important in detoxifying ROS produced by alloxan, with SOD reducing superoxide radicals to hydrogen peroxide and molecular oxygen, and catalase breaking down hydrogen peroxide into water and oxygen **(Lenzen, 2008)**. These reactions force Fenton reaction, and therefore, production of hydroxyl radical, the major damaging ROS related to β cell death, to halt **(Lenzen, 2008; Munday, 1988; Winterbourn and Munday, 1989)**. It was shown that excess of catalase production in liver hepatocytes, also possessing GLUT2 transporter and suitable for alloxan selective absorption, halted toxic effects of alloxan related to ROS production to the minimum **(Grankvist et al., 1979; Tiedge et al., 1997)**.

Data for G-SH and G-Per are shown in Table 3, with treatment having a significant influence on their values (p <0.05). The results for G-Red were non-significant (p >0.05, Table 3). Reduced glutathione is an important thiol-containing molecule, the presence of which causes alloxan- dialuronic acid cycling, resulting in the eventual production of ROS **(Lenzen, 2008)**. Alloxan-induced diabetes reduces levels of G-SH, and therefore, decreases reduced glutathione/oxidized glutathione ratio (G-SH/GSSG). However, increased levels of G-SH in β cells have a protective effect for glucokinase ("glucose sensor") inhibition by alloxan, as alloxan is kept in its oxidized form, dialuronic acid, incapable of reacting with thiol groups of glucokinase and deactivating it **(Ighodaro, Adeosun and Akinloye, 2017)**. Secondly, increased levels of G-SH cause activation of glutathione peroxidase, one of the enzymes capable of hydrogen peroxide deactivation. Progressed and established alloxan-induced diabetes reduced residual levels of G-SH in pancreatic tissue, though magnesium supplementation partially restored) G-SH levels to the value found in control animals (Table 3). A similar effect was observed with G-Per (Table 3), which also decomposes hydrogen peroxide. Glutathione reductase, which restores reduced glutathione levels through the reduction of its oxidized form, GSSG, using NADPH as reducing power, remained unchanged between treatments (Table 3).

Results for LDH and G6PD activity in pancreatic tissue after late established alloxan-induced diabetes are shown in Figure 6 and Figure 7. Treatment had a significant influence on both enzymes (p <0.05), with a significant decrease in their activities during late established diabetes (group D), and partial restoration in the case of magnesium supplementation after the onset of the disease in late progression. G6PD is a rate-limiting, first enzyme involved in the pentose phosphate pathway, which was shown to be inhibited during diabetes in the kidney cortex **(Xu, Osborne and Stanton, 2005)**. Pentose phosphate pathway generates extra reducing power in the form of NADPH, which can serve a substrate for glutathione reductase, which transforms oxidized GSSG into reduced G-SH, improving antioxidative "health" of the cells **(Xu, Osborne and Stanton, 2005)**. The LDH is the final enzyme in anaerobic oxidation of glucose, transforming pyruvate into lactate, and vice versa. Enzymatic activities of both LDH and G6PD in control untreated samples may serve as overall cell health state evaluation, and reduction in their activities may indicate impaired cellular health.

Several mechanisms may cause Mg^{2+} oral supplementation positive influence on pancreatic tissue, and specifically β cells, during experimentally induced diabetes. Magnesium complex with ATP activates glucokinase, an enzyme that serves as a "glucose sensor" as an initial step in cascade reactions of insulin release **(Gommers et al., 2016)**. Though it may happen even at a low sub-physiological level, some specific association between Mg-ATP and glucokinase may still exist **(Molnes et al., 2011)**, possibly decreasing alloxan inhibition of glucokinase. Intracellular magnesium concentrations inhibit L-type Ca^{2+} opening, avoiding massive Ca^{2+} influx inside cells, and "preserving" immediate insulin release and initial severe hypoglycemia caused by alloxan. Massive Ca^{2+} influx could also be one of the reasons for β cells death, besides ROS production **(Szkudelski, 2001)**. Magnesium serves as an antagonist of calcium, and more importantly, the ratio between Ca and Mg, not concentration levels themselves, causes insulin vesicle exocytosis **(Atwater et al., 1983)**. Additionally, magnesium dietary deficiency decreased antioxidant enzyme activities, namely, superoxide dismutase and catalase, in rat cardiac tissue, causing oxidative injury and explaining the pathogenesis of cardiac lesions in Mg-deficient rats **(Kumar and Shivakumar, 1997)**. A similar protective mechanism may be applied to magnesium influence on antioxidant enzymes in the pancreas. Contrary, although magnesium-deficient diet in rats caused decreased reduced glutathione levels in erythrocytes, implying its role in the maintenance of G-SH concentration to protect against oxidative damage in the erythrocyte membrane, the magnesium-deficient diet caused increased reduced glutathione levels in liver and kidneys, with no influence on other soft tissues **(Hsu, Rubenstein and Paleker, 1982)**. **Chaudhary, Boparai and Bansal (2007)** also showed an individual effect of low magnesium diet, as well as a combined effect of low magnesium with high sucrose diet on the development of oxidative stress in rats, namely, increase in plasma and liver lipid hydroperoxide levels, decrease in plasma reduced glutathione, decrease in liver superoxide dismutase, glutathione-S-transferase, and catalase enzyme activity.

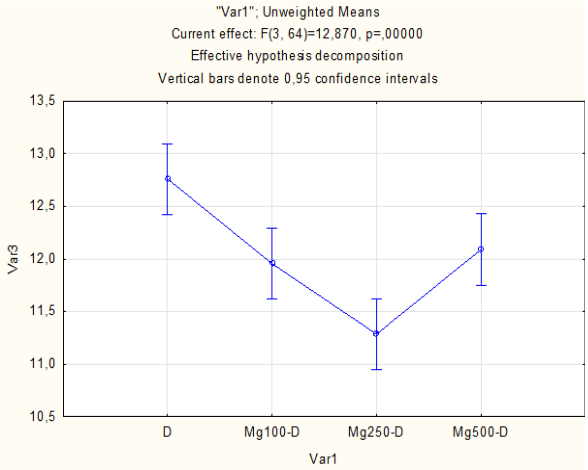

Figure 2 Influence of treatment (D, Mg100-D, Mg250-D, Mg500-D) on the mean value of fasting blood serum glucose levels on different days (day 21, 25, 27, 30). Error bars reflect standard error of mean. Adapted from Statistica ver 10.0.

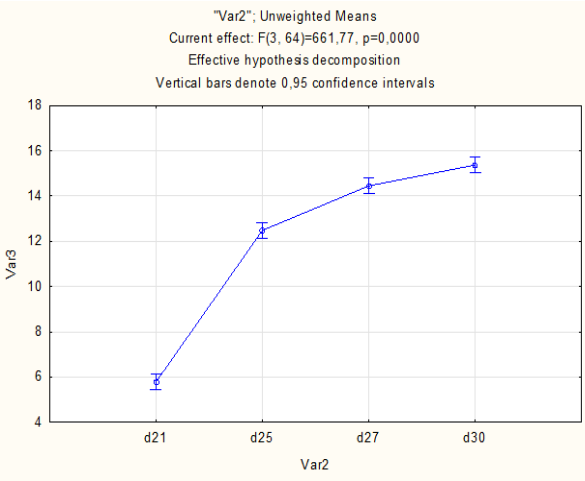

Figure 3 Influence of time factor (day 21, 25, 27, 30) on the mean value of fasting blood serum glucose levels in diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D). Error bars reflect standard error of mean. Adapted from Statistica ver 10.0.

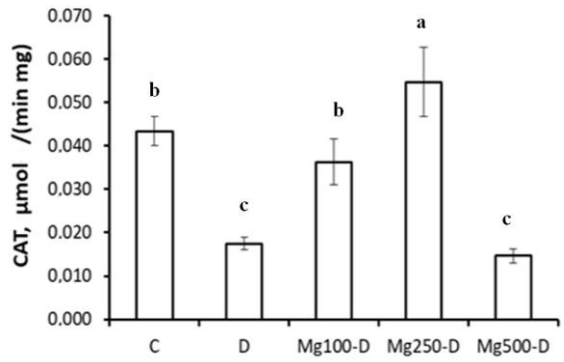

Figure 4 Mean values of pancreatic catalase (CAT) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

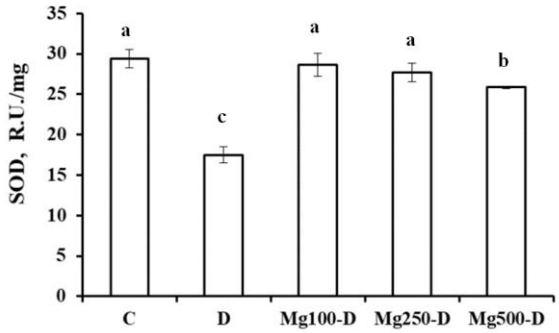

Figure 5 Mean values of pancreatic superoxide dismutase (SOD) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

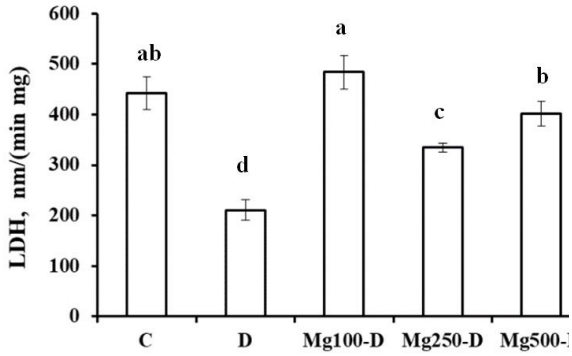

Figure 6 Mean values of pancreatic lactate dehydrogenase (LDH) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

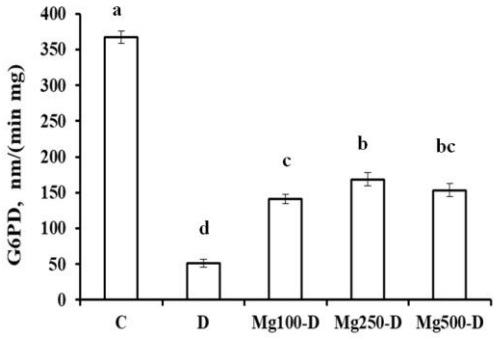

Figure 7 Mean values of pancreatic glucose-6-phosphate dehydrogenase (G6PD) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg-100D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

CONCLUSION

To summarize, daily oral magnesium supplementation at a dose of 250 $mg.kg^{-1}$ live weight in advance before alloxan-induced diabetes and within 10 days of the diabetic state improved fasting glucose levels in rats, though did not prevent the onset of diabetes. Oral supplementation with magnesium restored glucose metabolism enzymes levels, such as LDH and G6PD, in diabetic rat pancreas, compared to alloxan-induced diabetes alone, which suppressed their activity. Additionally, antioxidant system enzymes, namely, catalase, superoxide dismutase, glutathione peroxidase, and reduced glutathione levels, all improved in pancreatic tissue after magnesium administration in late-stage diabetic rats. Inconclusive results were obtained regarding lipid hydroperoxide, either due to technical issues or lack of influence of magnesium on the pancreas due to very late testing after the development of diabetes.

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THE EVALUATION OF EXTRACTION OF SOME NUT OILS USING SCREW PRESSING

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ABSTRACT

Today's consumers prefer low-sugar, low-calorie, natural, and so-called safe products. These trends are also reflected in nuts products and groceries. Globally, the European Union is the largest importing market of edible nuts. Considering the increasing demand for new sources of food, the importance becomes the efficiency of production. This study evaluates the influence of rotation speed in the extraction of almond nut, walnut, hazelnut, cashew nut, and peanut oils using screw pressing. In tested samples, the oil content was on average between 69.14 ±0.79% (walnut) and 46.7 ±1.45% (peanut). From the pressing of oils, it is seen that the oil yield decreased when pressing speed increased (from 30 rpm to 90 rpm, for example in walnut from 0.36 kg to 0.16 kg.h^{-1}) and that the oil sediment yield increased when speed increased (for example in hazelnut nut from 8.51% to 17.37%). The highest amount of oil yields had hazelnut with 3.03 ±0.05 kg.h^{-1}, then walnut with 2.05 ±0.02 kg.h^{-1}, almond nut with 2.34 ±0.05 kg.h^{-1}, peanut with 2.15 ±0.01 kg.h^{-1}, and finally cashew nut with 2.07 ±0.03 kg.h^{-1}.

Keywords: pressing time; oil yield; sediment yield; Soxhlet extraction; cake oil residue

INTRODUCTION

The European Union can produce only little amount of nuts within its area and therefore the EU is the largest importing market for edible nuts in the world, representing more than 40% of the total world imports **(CBI, 2018)**. Import in the year 2016 was 86,450 tons and volume is steadily increasing, but export was just 36,764 tons **(FAOSTAT, 2018)**.

Nuts are well known due to their high polyunsaturated fatty acids (PUFA) content and overly are very beneficial for health **(Christopoulos and Tsantili, 2015; Gharibzahedi et al., 2013; Kendall et al., 2011)**.

Consumers currently prefer low-sugar, low-calorie, natural, and so-called safe products which are the same for nuts groceries and products. The intentions of the food and confectionery industry are to exploit the full potential of nuts. Not only by adding them directly to products, but by making the most of their potential, for example by pressing oil and making use of cakes.

Oil from walnut kernel can be obtained in three ways, which are mechanical extraction (pressed oil), chemical or solvent extraction, and supercritical CO2 extraction **(Bhuiya et al., 2020; Zanqui et al., 2020; Alves et al., 2019; Singh and Bargale, 2000)**. The extraction by mechanical screw presses is one of the methods that are presently used and is typical for a lower proportion of collected oil **(Chapuis et al., 2014; Karaj and Müller, 2011)**. But benefits of screw pressing are to produce high-quality oil containing bioactive compounds, the possibility of using the cake residue, has relatively low initial and operation cost **(Al Juhaimi et al., 2018; Rabadán et al., 2018; Roncero et al., 2016; Alonge and Olaniyan, 2006; Alonge et al., 2003)**. The amount and quality of pressed oil are dependent on factors such as press time, temperature, the particular size of nuts, etc. **(Jokić et al., 2016; Gong and Pegg, 2015; Labuckas, Maestri and Lamarque, 2014; Teh and Birch, 2013)**.

The main objective of this study is to verify oil production from the imported seeds of hazelnut, walnut, almond nut, peanut, cashew nut and to compare its yield parameters.

Scientific hypothesis

The values of the pressing process for example yield, amount of sediment, or oil temperature of nut oils depend on the pressing speed.

MATERIAL AND METHODOLOGY

Nuts and kernels

Nuts and kernels (Figure 2), that were subject to this study, were vacuum-packed and purchased in the wholesale market. Before pressing, the water content in the nuts and kernels was assessed. They were ground in a stainless-steel mill with a pro-homogenization sample to

the fraction of size from 0 mm to 6 mm. Then, the material was pressed (Figure 1).

Determination of water content of nuts and kernels

The water content of nuts and kernels was determined by dehydration at 103 °C in a drying oven according to the **CSN EN ISO 665 (461025) (2001)**. The analysis was made on 5 g of grinded sample, weighted with an accuracy of 0.1 mg. Results are expressed as the ratio of water loss per gram of dried sample.

Screw press

The screw press type UNO FM 3F made by the Farmet Company in the Czech Republic was used for experimental measurements. The press allows precise adjustment of rpm from 0 to 200. The pressing device components are a matrix, 220 mm screw, head, heating mantle, nozzle holder, and nozzles of different in diameter (6, 8, and 10 mm). From the pre-test, the 8 mm nozzle was chosen as optimal. After the pressing process was the sediment separated and yield parameters of oils were determined. The oil temperature was measured directly on the press head every second by Testo 176 T4 – Temperature data logger (Testo SE & Co. KGaA, Germany) with stainless steel food probe.

Determination of the total fat content in the nuts, kernels, and cakes through extraction

To determine the total fat content, we used the Soxhlet extractor with hexane as a solvent. Crushing the walnut kernels always took place immediately before the oil extraction and from the pressed cakes directly after the pressing. For this purpose, the IKA MF 10 basic (IKA-Werke GmbH & Co. KG) on the sieve with an average of 3 mm was used. Precise cleaning of the grinder to avoid distorting results was always emphasized. The temperature of the extraction mixture was kept by the heating mantle closely around the boiling point of hexane (70 °C). Extraction was always carried out for 8 hours. Subsequently, the hexane was evaporated on the vacuum evaporator, type IKA RV 10 (IKA-Werke GmbH & Co. KG) control at the pressure of 200 kilopascals until the hexane was evaporated. After that, the pressure was lowered down to 60 kilopascals for another 2 hours at a constant temperature of 40 °C. The weight of total fat was then measured on the scales type KERN EG 2200-2NM (KERN & SOHN GmbH).

Statistical analysis

Analytical determinations were done in triplicate and data were reported as means ± standard deviation. Tukey's honestly significant difference (HSD) tests were conducted to determine the differences among which means that the statistical significance was declared at $p \leq 0.05$. The differences were analyzed only within the specific sample, not between the different samples. These statistical evaluation methods were applied using the computer software package "Statistica 12.0" (StatSoft Inc., USA).

RESULTS AND DISCUSSION

Table 1 and Table 2 shows the values of the main parameters. The average water content was at 3.95 ±1.21%, while the maximum content has hazelnut 5.8%, which corresponds to the values recommended for storage **(Poggetti et al. 2018; WCO HS 0802, 2017; Gong and Pegg, 2015; Christopoulos and Tsantili 2015)**. A most important parameter in the production of cold-pressed oils is oil content in nuts and kernels and the yield of producing oil. In samples, the oil content was on average between 46.7 ±1.45% until 69.14 ±0.79%.

From Table 1 and Table 2, it can be seen that the oil yield decreased when pressing speed increased **(Savoire, Lanoisellé and Vorobiev, 2013)**. This results confirmed Table 3 as a significant negative correlation between speed and yield of oil. The sediment is made by the imperfect separation of oil from the pressed material and represents an undesirable residual during the pressing process. It is also a signal that the press is not optimally set. Table 3 shows the significant correlation between speed and sediment in oils in Hazelnut, Walnut, and Almond nut, but not in Peanut and Cashew nut.

The results in Table 3 significantly proved that the amount of oil in pressed cakes and the efficiency of the press depends on the extraction parameters **(Rabadán et al., 2018; Jokić et al., 2016; Rombaut et al., 2015; Labuckas, Maestri and Lamarque, 2014; Teh and Birch, 2013)**, in our case on the rotation speed. Hourly performance increased with increasing speed, but not directly proportionally. At higher speeds, the efficiency of the press was lower due to the greater amount of sediment produced in the oil and the imperfect extraction of oil from the material. Therefore, the oil content in cakes increased. Oil temperature decreased in most of the cases while the speed was increasing. The evolution of the temperature of nut oils using screw pressing during oil extraction corresponds to the results of **Rabadán et al. (2018)**.

Many authors state, that in the pressed cakes, the leftover is between 5 – 31% of oil **(Gong and Pegg, 2015; Labuckas, Maestri and Lamarque, 2014; Acheheb, Aliouane and Ferradji, 2012)** and the value of sediment in pressed oil range from 7.95 to 17.57%.

The optimum speed for maximum oil yields for all samples was 30 rpm. But the higher speeds of the press increase the press capacity (yield of oil per hour), which is important for producers of oils. This is the reason why the setting of the pressing process is important according to the needs of each producer.

Hazelnut

Many authors reported that oil content in hazelnut ranged from 51 to 75% **(Kornsteiner-Krenn, Wanger and Elmadfa, 2013; Xu, Hanna and Josiah, 2007; Ebrahem et al. 1994)**. Our sample has a total fat content of 60.31 ±0.62%. The screw press makes it possible to achieve yield 50% **(Jokić et al., 2016)**, which was confirmed by this experiment and the total maximum weight of pressed oil was 51%, with 8.51% of sediment.

Table 1 The values of oil yield depended on speed.

Sample	Water content (%)	Total oil content (%)	Speed (rpm)	Weight of pressed oil (kg)	Weight of oil without sediment (kg)	Oil temperature (°C)
Hazelnut	5.8 ±0.57	60.31 ±0.62	30	0.51^{a} ±0.01	0.47^{c} ±0.02	49.2
			50	0.48^{a} ±0.01	0.41^{a} ±0.01	48.3
			70	0.45^{b} ±0.01	0.41^{a} ±0.00	43.7
			90	0.44^{b} ±0.02	0.36^{b} ±0.01	42.6
Walnut	4.2 ±0.66	69.14 ±0.79	30	0.42^{d} ±0.01	0.36^{a} ±0.01	47.6
			50	0.35^{c} ±0.01	0.33^{a} ±0.01	51.3
			70	0.29^{b} ±0.01	0.24^{c} ±0.03	42.1
			90	0.22^{a} ±0.01	0.16^{b} ±0.02	39.6
Almond nut	4.1 ±0.68	52.23 ±1.03	30	0.42^{d} ±0.01	0.40^{d} ±0.02	50.4
			50	0.39^{c} ±0.01	0.36^{c} ±0.01	50.1
			70	0.33^{b} ±0.01	0.31^{b} ±0.01	46.2
			90	0.29^{a} ±0.01	0.24^{a} ±0.01	37.7
Peanut	3.8 ±0.39	46.7 ±1.45	30	0.45^{d} ±0.01	0.41^{c} ±0.01	65.3
			50	0.42^{c} ±0.01	0.37^{b} ±0.02	62.0
			70	0.36^{b} ±0.01	0.31^{a} ±0.01	57.6
			90	0.31^{a} ±0.01	0.27^{a} ±0.03	56.2
Cashew nut	2.8 ±0.27	48.5 ±1.47	30	0.45^{d} ±0.01	0.40^{b} ±0.01	45.8
			50	0.43^{c} ±0.01	0.40^{b} ±0.02	43.6
			70	0.39^{b} ±0.01	0.34^{a} ±0.02	43.2
			90	0.36^{a} ±0.01	0.32^{a} ±0.02	39.4

Note: Alphabetical superscripts indicate significant differences (p <0.05) among values in columns for each sample.

Walnut

Many studies report, that walnut kernels have high oil content which varies from 52 to 72%. The fat content in tested raw material was determined at 69.14 ±0.73%, which is a rather higher percentage in comparison with other authors **(Kornsteiner-Krenn, Wagner and Elmadfa, 2013; Labuckas, Maestri and Lamarque, 2014)** and was the highest of tested samples. By pressing, the production of oil yield was 42%, with 12.94% of sediment. The results match with the results of **Gharibzahedi et al. (2013)** who indicates the highest yield crude pressed oil 34.9% and **Labuckas, Maestri and Lamarque (2014)** who states oil extraction in between 41.0 – 44.4% and a little lower than stated by **Sena-Moreno et al. (2016)** 57 ±3%.

Almond nut

Almond oil is extracted mainly from sweet almonds, which contain around 50% **(Fernandes et al., 2017)**, or 54.3% of oil **(Kornsteiner-Krenn, Wagner and Elmadfa, 2013)**. **Tungmunnithum et al. (2020), Prats (2000) and Roy, Mukherjee and Jana (2019)**, obtained yields of oil at 40 – 45%, which matches with our results 43% (with 6.98% of sediment) and **Sena-Moreno et al. (2016)** declare higher yield, almost 53 ±2%. This particular difference might be caused for example by the specific sample. To compare, our sample had total oil content 52.23%.

Peanut

Peanut seeds have oil content at 45 – 50% **(Kornsteiner-Krenn, Wagner and Elmadfa, 2013; Grosso et al., 1997)**. **Nader, Afif and Louka (2016)** state the yield production at 70% (when using the hydraulic press). At our experiment, we achieved the total maximum yield production of 45%, with 8.83% of sediment.

Cashew nut

The total oil content is on average between 45.2% **(Kornsteiner-Krenn, Wagner and Elmadfa., 2013)** to 52.08% **(Acheheb, Aliouane and Ferradji, 2012)**, with the highest oil yield 44.17% when the moisture content was 3.97%. The total maximum oil yield in this experiment was 45%, with 9.68% of sediment.

Table 2 The values of residues depended on speed.

Sample	Speed (rpm)	Amount of sediment in oil (%)	Weight of pressed cakes with oil (kg)	Weight of oil in pressed cakes (kg)	Amount of oil in pressed cakes (%)	Press capacity of oil ($kg.h^{-1}$)
Hazelnut	30	8.51^{a} ±1.29	0.43^{a} ±0.01	0.14^{b} ±0.02	31.59^{b} ±2.79	1.04^{b} ±0.04
	50	15.16^{bc} ±2.31	0.46^{a} ±0.01	0.19^{a} ±0.01	42.16^{a} ±2.13	1.90^{c} ±0.02
	70	10.65^{ab} ±1.58	0.49^{b} ±0.01	0.20^{a} ±0.01	40.60^{a} ±1.17	2.72^{a} ±0.02
	90	17.37^{c} ±3.00	0.50^{b} ±0.02	0.24^{c} ±0.01	47.83^{c} ±1.92	3.03^{a} ±0.05
Walnut	30	12.94^{a} ±0.91	0.54^{a} ±0.01	0.33^{a} ±0.01	60.80^{a} ±0.30	0.74^{a} ±0.05
	50	5.64^{a} ±4.74	0.61^{b} ±0.01	0.36^{a} ±0.01	59.39^{a} ±2.20	1.05^{b} ±0.05
	70	17.53^{ab} ±7.33	0.67^{c} ±0.01	0.45^{b} ±0.03	67.82^{b} ±3.21	1.80^{c} ±0.04
	90	27.74^{b} ±4.97	0.74^{d} ±0.01	0.53^{c} ±0.02	72.22^{b} ±1.30	2.05^{d} ±0.02
Almond nut	30	5.56^{a} ±2.76	0.54^{a} ±0.01	0.13^{a} ±0.02	23.28^{a} ±2.25	0.82^{a} ±0.02
	50	6.86^{a} ±3.88	0.57^{b} ±0.01	0.16^{b} ±0.01	28.35^{b} ±1.97	1.44^{b} ±0.05
	70	5.48^{a} ±4.17	0.63^{c} ±0.01	0.21^{c} ±0.01	33.48^{c} ±1.46	2.04^{c} ±0.05
	90	19.54^{b} ±1.44	0.67^{d} ±0.01	0.29^{d} ±0.01	43.05^{d} ±0.57	2.34^{d} ±0.05
Peanut	30	8.83^{a} ±4.25	0.51^{a} ±0.01	0.06^{b} ±0.01	11.18^{b} ±2.17	0.85^{a} ±0.04
	50	13.40^{a} ±2.83	0.54^{b} ±0.01	0.10^{c} ±0.02	18.63^{c} ±2.68	1.51^{b} ±0.03
	70	13.82^{a} ±4.42	0.60^{c} ±0.01	0.16^{a} ±0.01	26.12^{a} ±1.90	1.84^{c} ±0.03
	90	12.84^{a} ±6.55	0.65^{d} ±0.01	0.19^{a} ±0.03	29.87^{a} ±3.65	2.15^{d} ±0.01
Cashew nut	30	9.68^{a} ±2.48	0.52^{a} ±0.01	0.08^{a} ±0.01	15.58^{a} ±1.28	0.66^{a} ±0.03
	50	7.00^{a} ±4.62	0.54^{b} ±0.01	0.09^{a} ±0.02	16.24^{a} ±2.97	1.12^{b} ±0.06
	70	12.87^{a} ±2.90	0.58^{c} ±0.01	0.15^{b} ±0.02	24.84^{b} ±3.01	1.73^{c} ±0.06
	90	13.71^{a} ±7.15	0.61^{d} ±0.01	0.16^{b} ±0.02	26.58^{b} ±3.33	2.07^{d} ±0.03

Note: Alphabetical superscripts indicate significant differences (p <0.05) among values in columns for each sample.

Table 3 Correlation analysis between speed and production parameters in specific samples.

Sample		Weight of pressed oil (kg)	Amount of sediment in oil (%)	Amount of oil in pressed cakes (%)	Oil temperature (°C)	Press capacity of oil (kg/h)
Hazelnut		-0.941*	0.628*	0.868*	-0.959*	0.981*
Walnut		-0.994*	0.698*	0.870*	-0.813*	0.979*
Almond nut	**Speed (rpm)**	-0.986*	0.701*	0.967*	-0.916*	0.987*
Peanut		-0.982*	0.322	0.947*	-0.983*	0.979*
Cashew nut		-0.979*	0.432	0.856*	-0.952*	0.993*

Note: *Correlation is significant at the level 0.05.

Figure 1 Peanut oil pressing.

Figure 2 Samples of raw oils (from left peanut, walnut, almond, hazelnut).

CONCLUSION

The food and confectionery industry intend to exploit the full potential of nuts, for example by pressing oil and making use of cakes. The extraction by mechanical screw presses is one of the methods that is used for the production of cold-pressed oils.

In tested samples was oil content on average between 46.7 ±1.45% (peanut) until 69.14 ±0.79% (walnut). From the experiment, it is seen that the oil yield decreased when pressing speed increased (from 30 rpm to 90 rpm, for example in walnut from 0.42 kg to 0.22 $kg.kg^{-1}$) and that the oil sediment yield increased when speed increased (for example in almond nut from 5.56% to 19.54%).

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BIOLOGICALLY ACTIVE COMPOUNDS CONTAINED IN GRAPE POMACE

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ABSTRACT

A healthy lifestyle and gastronomic trends based on traditional and local foods accompanied by waste-free technologies are currently in the primary focus. One of the raw materials with properties in alignment with such requirements is grape pomace. This paper evaluates the antioxidant activity of grape pomace (which is homogenized into a brown powder) and selected commonly available commercial flours – wheat bread, rye plain, and rye whole grain flour – using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and total polyphenol content method, where was used Folin-Ciocalteaure agent and then it was determined by spectrophotometric method (the measure of absorbance). The total amount of polyphenols in grape pomace was measured of 47.94 mg GAE.g^{-1}, but the value 0.27 mg GAE.g^{-1} was measured in wheat bread flour. Grape pomace performed the antioxidant activity of 57.45 mg AAE.g^{-1}, whereas wheat bread flour of only 0.21 mg AAE.g^{-1} Compared to selected commercial flours, the total amount of polyphenols in grape pomace was up to 150 times higher and the ratio of antioxidant activity between grape pomace and wheat bread flour was even more than 280 times higher. This makes it possible to fortify commercial, commonly available flours with different amount of grape pomace so that products with a higher amount of biologically active substances can be prepared. Another benefit could be a reduction in health risks and a contribution to improving consumer health.

Keywords: grape pomace; polyphenolic compounds; antioxidant activity; flour

INTRODUCTION

The interest in healthy and balanced nutrition has been increasing. The society is striving to find suitable food beneficial to the human body – a diet rich in vitamins and minerals. The WHO recommends 600 g of fruit and vegetable, including cooked vegetables, as a daily intake **(WHO, 2013)**. The ratio between fruit and vegetable should be 1:2 **(The Czech Society for Nutrition, 2012)**. Unfortunately, in many European countries, their income is lower. However, fruit and vegetable are rich in biologically active substances **(Juriková et al., 2016)**. These include antioxidants, such as vitamins C, E, provitamin A, minerals with antioxidant properties, polyphenols, tannins, and other groups of substances that can reduce health risks and contribute to health enhancement **(Salter, Wiseman and Tucker, 2012; Tomás-Barberán and Gil, 2008)**. One source of these substances may be grapevine and products from it **(Katalinić et al., 2010; Kim et al., 2011; Liang et al., 2014; Stockham et al., 2013)**.

Grapevine is one of the best known and most widespread fruits in the world **(Yang et al., 2009; Yang, Martinson and Liu, 2009; Soto, Brown and Ross, 2012)** and includes four species: the European type (*Vitis vinifera* L.), American grape type (*Vitis labrusca* L.), Muscadine type (*Vitis rotundifolia* Michx.) and Amurensis (*Vitis amurensis*). Out of these, the European type has spread worldwide and accounts for 71% of total grape production in the wine industry **(Yang, Martinson and Liu, 2009)**. An example of producing of grapes can be mentioned in Brazil, where the production was approximately 534 million kg in the year 2009. This number increased by 24%, which means that the number was 709 million kg two years later (in the year 2011). Logically, when the production of grapes increased, automatically the amount of grape pomace increased as well **(Casagrande et al., 2019)**. Grapes serve as a suitable source of phenolic compounds with antioxidant effects, such as anthocyanins, flavonoids, and resveratrol **(Yang, Martinson and Liu, 2009; Hernández-Salinas et al., 2015)**.

It is a fruit consumed all over the world. It can be eaten in a form of fresh table fruit and also in a form of diverse processed products including wine, juice **(Kim et al., 2017)**, grape flour, or grape pomace. Grape pomace, dried and homogenized grape seeds and skin, is a by-product of wine production applied for further processing. It can be used as a substrate to produce alcoholic beverages due to sufficient sugar content. The most famous liquor prepared

from grape pomace is Italian Grappa. In France, brandy originated from grape pomace is known as Marc, in Spain it is named De orujo and in Portugal Bagaceira **(Piras, 2008; Gasnier, 2005)**. Furthermore, the inedible components such as seeds and skins can be used as a food additive, feed, fertilizer, seed source for oil production, or fuel. Therefore, grape pomace is considered to be a favorable alternative product allowing waste-free management. Grape pomace is rich in polyphenols, especially flavonoids such as gallic acid, catechin, and epicatechin **(Özvural and Vural, 2014)**.

Polyphenolic compounds are plant secondary metabolites present in plant foods and they can affect human health **(Ziauddeen et al., 2019; Pinto and Santos, 2017; Zehiroglu and Sarikaya, 2019)**. It is a complex group of phytochemical compounds ranging from simple phenols and phenolic acids to high molecular weight polymer structures (e.g. hydrolyzed and condensed tannins) **(Ziauddeen et al., 2019; Zamora-Ros et al., 2016)**. From a chemical point of view, it is a diverse and heterogeneous group of organic compounds **(Llobera and Cañellas, 2007)**. Their distribution in different parts of plants is diverse. Commonly, they are present in outer layers of fibrous plant material; insoluble substances in the cell walls, and soluble in vacuoles **(Pandey and Rizvi, 2009)**. The number of phenolics which has been found in plants is over 8000. The particular profile of polyphenols depends also on specific cultivar within a species. An example can be given grape varieties where the composition can depend on factors, such as geographical region, soil composition, or terroir. This is the reason why are different polyphenolic compositions but also the success of growing specific species of wine **(Bakota et al., 2015)**.

As a by-product, grape pomace is an inexpensive source of polyphenolic antioxidants **(Bakota et al., 2015)** attracting growing interest as it offers a wide range of usable products in the food, cosmetic and pharmaceutical industries **(Milella et al., 2019)**. Several phenolic compounds have a role in preventing lifestyle diseases, such as cardiovascular diseases, diabetes mellitus type 2, neurodegeneration, and certain types of cancer **(Bončíková et al., 2012; Ziauddeen et al., 2019)**.

The process of obtaining polyphenols from fruit or vegetable is usually extraction. It can be for example Soxhlet extraction, maceration, or hydrostillation. Nowadays there are also new non-conventional methods, due to the effort to use a method that is environmentally friendly to compare to the classical method. An example can be ultrasound, microwave, and pressure-assisted extractions (it can be applied alone or together with various solvents for the sake of reducing solvent requirements and the energy) **(Milella et al., 2019)**.

Furthermore, apart from phenolic compounds with antioxidant effects, grape pomace contains other antioxidants neutralizing free radicals. In the human body, free radicals may harm compounds altering cell membranes, cause cell damage and cell inflammation, promote abnormal cell growth including certain types of cancer and even cause cell death. Free radicals are formed in the body in natural metabolic processes. In their formation, exogenous factors, including ultraviolet light, radiation, fumes, and air pollution, play an important role **(Krishnaswamy et al., 2013)**. Antioxidants, comprising phenolic compounds, flavonoids, and carotenoids, can reduce the risk of oxidative damage by converting free radicals into inactive molecules **(Štípek et al., 2000)**.

These beneficial properties of grape pomace are in accordance with current nutritional trends emphasizing the application of traditional, local and organic food in compliance with waste-free management **(Burg, Masan and Ludin, 2017; Makris et al., 2008)**.

Due to the fear of the unfavorable effects and the safety of synthetic antioxidants, the studies started to be more interested in studies that were focused on natural products. Namely, it was for example herbs, vegetables, fruits, or agro-industrial waste. The reason for attention on these products is because they are rich in polyphenols and they can be used instead of synthetic antioxidants that are used in the pharmaceutical, food, or cosmetic industry **(Casagrande et al., 2019)**.

It is assumed that polyphenol content is about 60 – 70% in grape seeds, 30% in the skin, and merely 6% in the flesh **(Kim et al., 2017)**.

Homogenized grape pomace has reached the market and allows fortification of common commercial flours or their complete replacement. The purpose of fortification is to increase the content of biologically active substances. First, it is necessary to identify the original content of biologically active substances in the individual basic materials.

This paper aims to compare polyphenolic substances in grape pomace and their antioxidant effects with commercial, commonly available flours on the market.

Scientific hypothesis

Hypothesis No. 1: The total content of polyphenolic substances and antioxidants in grape pomace is higher than in the commercial flours.

MATERIAL AND METHODOLOGY

Material for analysis

The analysis employed material samples from grape pomace (Figure 1), wheat bread flour, rye flour, and rye wholemeal flour. A presented grape pomace sample came from viticulture Ludwig, vineyard track ”Zbavce”, village Němčičky, region Velkopavlovická, area Moravia. It was homogenized from grapes of the Riesling variety which was manually harvested in October 2018. Next, the grapes were processed using a time-driven horizontal press. Drying and grinding were performed at 70 °C. The grinding was performed until the grape pomace reached similar size as the flours which were bought for the analysis.

The flours were bought in a supermarket as a commonly available commercial material:

– wheat bread flour – wheat flour “Babiččina volba”, GoodMills Česko inc., Prague, the Czech Republic, with the following nutritional values per 100 g: energy 1461 kJ/345 kcal, fats 1.7 g, of which saturated fatty acids 0.2 g, carbohydrates 69 g, of which sugar 2.0 g, fiber 3.1 g, proteins 12 g, salt <0.01 g;

Figure 1 The grape pomace sample.

– rye plain flour – rye plain dark flour "Babiččina volba" GoodMills Česko inc., Prague, the Czech Republic, with the following nutritional values per 100 g: energy 1435 kJ/339 kcal, fats 1.0 g of which saturated fatty acids 0.2 g, carbohydrates 70 g, of which sugars 6.0 g, fiber 9.0 g, proteins 8.0 g, salt <0.01 g;
– rye wholemeal flour – "Pernerka" rye wholemeal fine flour, Mlýn Perner, Svijany, the Czech Republic, with the following nutritional values per 100 g: energy value 1449 kJ/342 kcal, fats 1.3 g of which saturated fatty acids 0.13 g, carbohydrates 72.1 g, of which sugars 1.8 g, proteins 6.1 g, salt 0 g.

An extract (5 g of sample extracted in 50 mL of methanol (MERCI, s.r.o., Brno, the Czech Republic)) was prepared from all flour samples and used for further determination.

Total phenolic content analysis

To determine the total phenolic content (TPC) Folin-Ciocalteaure agent (Sigma Aldrich, the Czech Republic) was used. The extract of the amount of 0.1 mL was mixed with water in a 10 mL volumetric flask. Then, 0.5 mL of Folin-Ciocalteaure agent and 1.5 mL of 20% solution of Na_2CO_3 (Sigma Aldrich, the Czech Republic) were added to the solution. Specord 50 Plus (Analytik Jena, Jena, Germany) was used to measure absorbance at a wavelength of 765 nm. Pure water was used as a reference **(Thaipong et al., 2006)**. To express the results, unit milligrams of Gallic acid (GAE) per grams of fresh mass (FM) were used. TPC was measured four times.

Total antioxidant capacity analysis

Total antioxidant capacity (TAC) was established applying DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) (Sigma Aldrich, the Czech Republic) assay in accordance with the study by **Brand-Williams, Cuvelier, and Berset (1995)**.

The stock solution was prepared by the solution of 24 mg of DPPH in 100 mL of methanol (MERCI, s.r.o., Brno, the Czech Republic) which was stored at -20 °C until it was needed. The absorbance of DPPH radical without any added extract was recorded and corrected every day. The sample solution used for measuring was prepared by mixing 10 mL of the stock solution with 45 mL of methanol to obtain the absorbance of 1.1 ±0.02 units at 515 nm using spectrophotometer Specord 50 Plus (Analytik Jena, Jena, Germany). The extract (210 μL) was allowed to react with a 4 mL DPPH solution for 1 hour in the dark. Then, the absorbance was recorded at a wavelength of 515 nm. TAC was measured three times.

For the calculation of antioxidant capacity, a decrease of the absorbance value was used following the formula:

Antioxidant capacity (%) = $(A_0 - A_i/A_0) \times 100$,

where A_0 is the absorbance of a blank without the sample and A_i is the absorbance of the mixture containing the sample. The calculated antioxidant capacity was converted using a calibration curve of the standard and expressed in ascorbic acid equivalents (AAE) **(Vasantha Rupasinghe, Jayasankar and Lay, 2006)**.

Statistical analysis

Excel 2013 (Microsoft Corporation, USA) and STATISTICA CZ version 12 (StatSoft, Inc., USA) were used for data analysis. The results were expressed by mean ± standard deviation. The comparison of TPC and total antioxidant activity (TAA) of grape pomace with those of flour samples were calculated by non-parametric tests – Kruskal-Walllis test Wald-Wolfowitz test, Kolmogorov-Smirnov test and Mann-Whitney U Test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

The comparison of total polyphenol content of grape pomace and selected commercial, commonly available flours are shown in Table 1. The lowest value of polyphenols was established in wheat bread flour with a value of 0.27 $mg.g^{-1}$. Rye flour showed a slightly higher value in both plain and whole grain flour. The total amount of polyphenols in grape pomace was measured to be 150 times higher, namely 47.94 $mg.g^{-1}$. This indicates a statistically significant difference between grape pomace and commercial flours.

Due to the non-compliance with the prerequisites for the ANOVA type statistical evaluation, the Kruskal-Walllis test based only on the order of the individual samples had to be applied to compare obtained data as can be seen in Table 2. For this reason, a statistically significant difference was observed only between grape pomace and wheat bread flour, although the measured values indicated a statistically significant difference between grape pomace and all the commercial flours. In case that only two individual samples are compared, there was a statistically significant difference ($p < 0.05$) between all flour samples both using non-parametric tests (Wald-Wolfowitz test, Kolmogorov-Smirnov test, Mann-Whitney U Test) and using the parametric Welch t-test. However, the homogeneity condition is not met in this test.

Published studies devoted to the issue of this study have revealed a wide range of values. For instance, **Costa et al. (2019)** declared the value of 4.7 ±0.0 mg $GAE.g^{-1}$ for grape pomace of red wine from Brazil. In contrast, **Theagarajan et al. (2019)** established the total content of polyphenols in dry compacts from ripe red grapes of the Muscat variety grown in India at the amount of 280.6 ±3 mg $GAE.g^{-1}$. **Chamorro et al. (2012)** determined the total content of extractable polyphenols in grape seeds

from France of 296 ±9 mg GAE.g^{-1} in dry material and 23.6 ±0.8 mg GAE.g^{-1} in grape pomace originated from Spain. Similar values of total polyphenol content as found in this study are documented in the article by **Casagrande et al. (2019)** in the range of 17.91 to 35.10 mg GAE.g^{-1}. Considering grape seeds from different cultivars, **Sung and Lee (2010)** reported polyphenol levels varying from 7.92 mg GAE.g^{-1} to 43.69 mg GAE.g^{-1}. **Rockenbach et al. (2011)** measured the total phenolic content in various wines and the measured range is 32.62 – 74.75 mg GAE.g^{-1}.

In a comparison of different types of flour, **Li et al. (2015)** measured different coloured wheat, where the values were in the range of 0.51 to 0.66 mg GAE.g^{-1}. **Ragaee, Abdel-Aal and Noaman (2006)** state that in wheat flours and whole grain cereals is the range of 0.50 to 4.22 mg GAE.g^{-1}, which is similar to the value of wheat flour which was measured in this paper, and again lower than the values for grape pomace.

For gluten-free flours, in the paper **Rocchetti et al. (2019)** the values were similar too – the total polyphenolic content was in the range 0.52 to 5.00 mg GAE.g^{-1}. **Alvarez-Jubete et al. (2010)** measured the polyphenols of seeds of amaranth, quinoa, buckwheat, and wheat in the range 0.21 – 3.23 mg GAE.g^{-1} dry-weight basis.

Interesting results for comparing were also analyzed in **Ky and Teissedre (2015)** where was measured total phenol content in grape pomace seed and skin extracts. The measured amount was 128.22 – 215.93 mg GAE g^{-1} of dry weight in grape seed pomace extract and 71.88 – 196.71 mg GAE g^{-1} of dry weight in grape pomace skin extracts when for the measuring was used the extraction method which is appropriate for the preparation of edible extracts.

For comparison, there are some studies where were measured the amounts of polyphenols in wines, where the unit was in mg GAE.L^{-1}. In the study **Boussenna et al. (2016)** three different grape pomace extracts were used. The amount of polyphenols was in the range of 140 to 400 mg GAE g^{-1} grape pomace extract. **Snopek et al. (2018)** were analyzing wine where the values for white wine were 203.06 – 678.78 mg GAE.L^{-1} and for red wine 905.21 – 1349.12 mg GAE.L^{-1}. The average amount of measured wine in **Bajčan et al. (2016)** was 2,424 mg GAE.L^{-1}. **Bajčan, Čéryová and Tomáš (2012)** measured range 1579 – 2734 mg GAE.L^{-1} in Blaufränkisch. The range of wine from various Slovak vineyard areas which was measured by **Bajčan et al. (2015)** was 2064 – 4274 mg GAE.L^{-1}. In the study **Lapčíková, Lapčík and Hupková (2017)** was measured the range 549.44 to 2832.78 mg GAE.L^{-1} for a variety of wines. The higher values were measured by **Garaguso and Nardini (2015)**, where the mean was 4417 mg GAE.L^{-1} for organic wines and 4225 mg GAE.L^{-1} for conventional wines. **Faitová et al. (2004)** measured the amount of total polyphenolic content in twelve bottles of Traminer, and the values were in the range 257.7 – 282.5 mg GAE.L^{-1}. The study **Ivanova-Petropulos et al. (2015)** is showing that the differences can be seen in the same country (Macedonia) but different regions, where the values were measured in the range of 1394 to 3097 mg.L^{-1}. The wide range is shown in **Fanzone et al. (2012)** where were used thirty red wines from the 2010 vintage, the values were 1585.6 – 4203.2 mg.L^{-1}.

Kondrashov et al. (2009) measured different varieties of Merlot and Cabernet wine, and the total phenolic content was in the range from 1447 to 2414 mg GAE.L^{-1}. **Burin et al. (2010)** measured Cabernet as well and the results of their measurings are in the range of 1750.9 – 2424.1 mg GAE.L^{-1}. **Yoo et al. (2011)** reported wide range, namely 1417.14 – 3588.57 mg GAE.L^{-1} for Cabernet Sauvignon and 1181.43 – 3288.57 mg GAE.L^{-1}

Table 1 Total Polyphenol Content in individual samples.

Flour	Total Polyphenol Content	
	M **(mg GAE.g^{-1})**	***SD*** **(mg GAE.g^{-1})**
wheat bread	0.27	0.01
rye plain	0.40	0.01
rye wholemeal	0.51	0.01
grape pomace	47.94	0.11

Note: *M* – mean, *SD* – standard deviation.

Table 2 Total phenolic content (TPC) in individual analysed samples – Multiple comparison of *p* values.

Flour	wheat bread	rye plain	rye wholemeal	grape pomace
wheat bread		*p* >0.05	*p* >0.05	***p* <0.05**
rye plain	*p* >0.05		*p* >0.05	*p* >0.05
rye wholemeal	*p* >0.05	*p* >0.05		*p* >0.05
grape pomace	***p* <0.05**	*p* >0.05	*p* >0.05	

Table 3 Total antioxidant activity (TAA) in individual analysed samples.

Flour	Total antioxidant activity	
	M **(mg AAE.g^{-1})**	***SD*** **(mg AAE.g^{-1})**
wheat bread	0.21	0.06
rye plain	0.41	0.06
rye wholemeal	0.63	0.03
grape pomace	57.45	0.42

Note: *M* – mean, *SD* – standard deviation.

Table 4 Total antioxidant activity (TAC) in individual samples – Multiple comparison of *p* values.

Flour	wheat bread	rye plain	rye wholemeal	grape pomace
wheat bread		*p* >0.05	*p* >0.05	***p* <0.05**
rye plain	*p* >0.05		*p* >0.05	*p* >0.05
rye wholemeal	*p* >0.05	*p* >0.05		*p* >0.05
grape pomace	***p* <0.05**	*p* >0.05	*p* >0.05	

for Shiraz wines. **Paixão et al. (2007)** compared five red wines, one rosé wine and five white wines and the result was in the range 282 – 1936 mg GAE.L^{-1}. As can be seen, there are differences between wine (the liquid product of grapes) as well, which shows the variety of polyphenols which can be seen in the different types of grapes, that were grown in the different conditions.

The amount of TPC is affected by a number of factors, so the results presented in this article may be different compared to the others. The content of polyphenols can be affected by environmental factors such as soil type, sunlight, climate, etc., TPC is further affected by storage, during which polyphenols oxidize easily. Concentrations of phenolic acids generally decrease during maturation, while concentrations of anthocyanins increase. Another parameter that affects the content of polyphenols is culinary treatment – grinding of plant tissues degrades polyphenols and maceration can increase the content of polyphenols due to diffusion in the juice **(Manach et al., 2004; Pandey and Rizvi, 2009)**.

Table 3 summarizes antioxidant activities which were up to 280 times higher in grape pomace than in other commercial flours. Grape pomace performed the antioxidant activity of 57.45 AAE $mg.g^{-1}$, whereas wheat bread flour of only 0.21 AAE $mg.g^{-1}$. Samples of rye flour differed from wheat bread flour by only 0.20 AAE $mg.g^{-1}$, which is a negligible difference if compared to grape pomace.

Similarly to the determination of the total amount of polyphenols, it was not possible to apply the ANOVA test. Therefore, a comparison of the obtained data was performed using the Kruskal-Walllis test as displayed in Table 4. The results follow the same trend as for total polyphenols content. As with the TPC determination, non-parametric tests (Wald-Wolfowitz test, Kolmogorov-Smirnov test, Mann-Whitney U test) and parametric Welch t-test between individual pairs of samples were performed again. There was a statistically significant difference (p <0.05) between all pairs in all tests.

Concerning grape seeds from different cultivars, **Sung and Lee (2010)** reported antioxidant activity ranging from 28.2 ("Agawam") to 121.2 ("Cabernet Sauvignon") µmol Trolox.g^{-1}. **Costa et al. (2019)** documented 160.9 ±22.3 µmol Trolox.g^{-1} in unprocessed grape seeds and 105.5 ±2.0 µmol Trolox.g^{-1} in defatted grape seeds. Grapes pomace samples of Château Roslane (Meknes, Morocco) were employed in the study by **Aziz et al. (2019)** publishing the values ranging from 0.12 ±0.0 to 0.23 ±0.0 mmol Trolox.g^{-1} dry weight.

Even though more studies are examining this issue, data comparison is very problematic as different methodologies and their modifications, different grape varieties, and sample processing techniques of TPC and TAA determination were applied. It is essential to consider specifics of biological material that may be influenced by the ambient conditions (climate, temperature, sunshine), cultivation technologies (soil fertilization, harvest time, harvesting method), and by post-harvest processing and storage.

For future practical applications, it would be appropriate to monitor TAA and TPC in the mixtures of common commercial meals and grape pomace in various ratios to enable fortification of traditional flour with a small percentage of grape flour. Furthermore, it is necessary to identify specific characteristics of such mixtures, including sensory properties and leavening, and subsequently, to examine properties in baked goods, such as shelf life, to distinguish their mutual influences.

CONCLUSION

This research has examined the content of antioxidants and polyphenolic substances in grape pomace which were gained from the local farmer and it was thoroughly compared with three different commercials, commonly available flours, where two of them were from one company and the last one was from the different one for more valuable comparison. It evaluates differences in existing products and products containing non-traditional alternatives to commercial ones. The results indicate that grape pomace performed magnitude higher values of both polyphenolic contents and antioxidant properties than commercial flours.

As this study has revealed, grape pomace provides a diverse array of applications in the food industry; it plays a role as a food additive improving sensory and technological properties and works as a dietary supplement reducing the risk of civilization diseases including cardiovascular diseases, diabetes mellitus, and obesity. Newly, further advantageous attributes still required to investigate more is the possibility to employ grape pomace in gluten-free and lactose-free products.

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DEVELOPMENT OF AN INTEGRATED FOOD QUALITY MANAGEMENT SYSTEM

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ABSTRACT
The high-speed growth in the global population has resulted in a deficit of foods, which has stimulated the development of technologies for planting agricultural products and fattening domestic animals. However, these processes are supplemented in many cases by worsening of the quality of foods and their pollution by foreign substances. To guarantee the proper quality and safety of foodstuffs for health, the International Organization for Standardization developed the standards ISO 9001:2015 and ISO 22000:2018. At the same time, businesses fabricating foods, especially meat-based ones, have to observe the norms of the international standard ISO 14001:2015. Finally, because treatments of raw materials and ingredients used in food products contain in many cases substances harmful for health, enterprises must introduce the norms of standard ISO 45001:2018. To simplify management processes, enterprises introduce so-called 'integrated management systems'. This study proposes one variant of such a system recommended for use in food-producing organizations including those that treat raw meat and produce products based on its use.

Keywords: quality management system; integrated management system; quality; safety; food; food safety; quality standard

INTRODUCTION
The world population is currently 7.7 billion people and continues to increase. The United Nations Population Division estimates that it will be about 9 billion in 2042 and may rise to 16 billion by 2100.

As a consequence of such fast growth, the problem of ensuring humankind has an adequate quantity of foodstuffs originated as early as the 1940s. This phenomenon prompted the beginning of the so-called 'green revolution' in agriculture, i.e. the dramatic change in technologies of planting and of fattening domestic animals **(Hazell, 2009)**. The principal steps in such evolution were the introduction of the practice of irrigation and the use of pesticides and chemical fertilizers which permitted a doubling of cereal productivity to 1985. However, solely increasing output is not sufficient to solve the problem of supplying foodstuffs because it is not the only task of the food industry. Enterprises must also produce products safe for the health of consumers. They must also observe the norms of the technologies of farming and reprocessing of crops, protection of the environment, and guaranteeing safe labour conditions for production personnel.

The current problem in the food industry is that intensification of farming practice has resulted in decreasing water availability, infringement of water intake schedules, and salification and desertification of considerable areas of fertile soils. The result of wide-ranging use of mineral fertilizers and chemical means of protecting plants (aromatic heterocyclic compounds of chlorine and phosphorus) is worsening human health and is the origin of problems of ecological character. Hence the World Health Organization (WHO) adopted a resolution that recognizes the problem of food safety as crucial in protecting human health because food pollutants of chemical and radiological character, bacteria, viruses, and vermin provoke the origin of more sicknesses **(WHO, 2014)**. It has been shown that consumption of poor-quality food and water results in the death of up to 2 million people worldwide each year **(Brijnath, Butler and McMichael, 2014)**.

To decrease the rate of accidental deaths, the WHO recommends the introduction of the following principles of alimentary hygiene: a) consumption of only safe water and raw materials in the fabrication of foods, b) protection of raw materials and ingredients against contact with insects, rodents and vermin, c) prevention of contamination of products by harmful substances and pathogens transferred by people, domestic animals, and vermin, d) placing raw products and finished ones in separate places to prevent their cross-contamination, e) cooking of foods at scheduled temperatures and for a time sufficient to annihilate harmful microorganisms, f) storage of finished foods at the scheduled temperature **(WHO, 2018)**.

Thus, the issue of food safety and quality control is an important area of research in Ukraine and across the world. A similar issue has been raised and studied in Russia **(Tsaregorodtseva et al., 2020)**. The authors argue for the use of the best practices of EU countries in Russia, to improve its own regulatory and legislative framework to empower the elimination of threats to the safety of food raw materials and food products. This will allow the reduction to an acceptable level of the risks of contaminating food products at all stages from production to sales, thereby guaranteeing the end-user safety of food products.

Scientific hypothesis

As a result of a systematic analysis of theoretical research, we will have formulated and implemented the principles of a systematic approach to the development of an integrated management system for product quality and safety of meat processing enterprises in Ukraine. This approach will provide an opportunity to develop a risk and critical control point (HACCP) plan for meat processing plants, following the example of the world's best practices. Based on a comprehensive approach to the systematization of international standards, an integrated system of product quality management and the safety of meat processing enterprises operating in conditions of minimal environmental damage will be developed. Thanks to the introduction of an integrated quality system, the overall level of food safety in Ukraine will be increased.

MATERIAL AND METHODOLOGY

Theoretical research methods were used in the work: a comprehensive study of the provisions of legislative, regulatory, and normative documents on the criteria for compliance of food industry enterprises with the norms of product quality and safety, the proper state of the environment, and safe working conditions for staff.

Theoretical methods of research

Methods of literary source and document analysis, induction and deduction, analysis and synthesis **(Hennink, Hutter and Bailey, 2020)** were used, which made it possible to determine the principles of a systematic approach to the development of an integrated management system for product quality and safety of meat processing enterprises in Ukraine.

International documents as a subject of analysis:

1) HACCP meat processing plant; good practices of hygiene (GHP), manufacture (GMP), and distribution (GDP).

2) **ISO 45001:2018 (2018)**, **ISO 9001:2015 (2015)**, **ISO 14001:2015 (2015)**, **ISO 22000:2018 (2018)**, **ISO 22004:2014 (2014),** and the national standards of different countries.

Normative documents on the problems of industrial sanitation and safety of work on production lines, as a subject of analysis:

Fire safety rules according to **GOST (1992)**; air quality standards of the working area according to **GOST (1989)**; vibration safety standards according to **DSTU GOST (2009a)**; norms of fire and explosion safety of static electricity according to **GOST (1993)**; safety standards for handling technological equipment according to **GOST (1991)**; norms of safe arrangement of workplaces according to **DSTU GOST (2009b)**; safety standards for technological processes according to **GOST (2014)**; norms of arrangement of supply and exhaust ventilation systems according to **DSTU (2010)**; norms of industrial noise according to the state sanitary norms of **LTO (1999a)**; vibration norms in the organization of production activities according to the state sanitary norms **LTO (1999b)**; microclimate parameters in production facilities according to the state sanitary norms of **LTO (1999c)**; parameters of heating, ventilation and conditioning according to sanitary norms and rules of **SNiP 2.04.05-91 *U (1997)**; the procedure for washing and disinfection of industrial and domestic premises according to the instructions **I 123-5/990-11-84 (1984)**; fire safety rules according to the norms of the document **NAPB (2004)**; lighting standards according to the requirements of the state building code **DBN (2006)**; norms of providing personnel with special clothes, special shoes and personal protective equipment according to the norms of the document **NPAOP (2008)**; the procedure for training and retraining of staff according to the requirements of the document **NPAOP (2005)**; rules of work on electrical installations according to the requirements of the document **NPAOP (1998)**.

Statistical analysis

The classic methods of Deming and Juran were used, which were developed during the whole period of formation of quality management and which have remained relevant today. Their essence is to study the development of methods of quality planning and statistical analysis **(Anderson et al., 1995)**.

RESULTS AND DISCUSSION

The first step in realizing a system of management for the safety of foodstuffs was the introduction by businesspeople of the principles of the HACCP system **(Hulebak and Schlosser, 2002; Lozova, 2019)** and GHP, GMP and GDP, as well as sanitary norms for maintaining equipment, buildings, and installations normalized by the international standard of food safety **ISO 22000:2018 (2018)**. This document normalizes the order of work by identifying risks and verifying the conformity of the index quality of production and its ingredients with their regulated norms (Table 1).

Table 1 Foodstuff quality control procedures.

Stage of work	Procedure	Result
Inspection	Control of conformity of procedures of work with the recommended norms	Working group reaches specified result
Control	Evaluation of results of work and/or comparing quality indices of samples in development with the basic norms	Quality control indices of new products reach their normalized values
Confirmation	Attestation of facilities by manufacture of new products	Production is safe and its quality conforms with the needs of specific groups of the population (children, adults with specified needs, etc.)

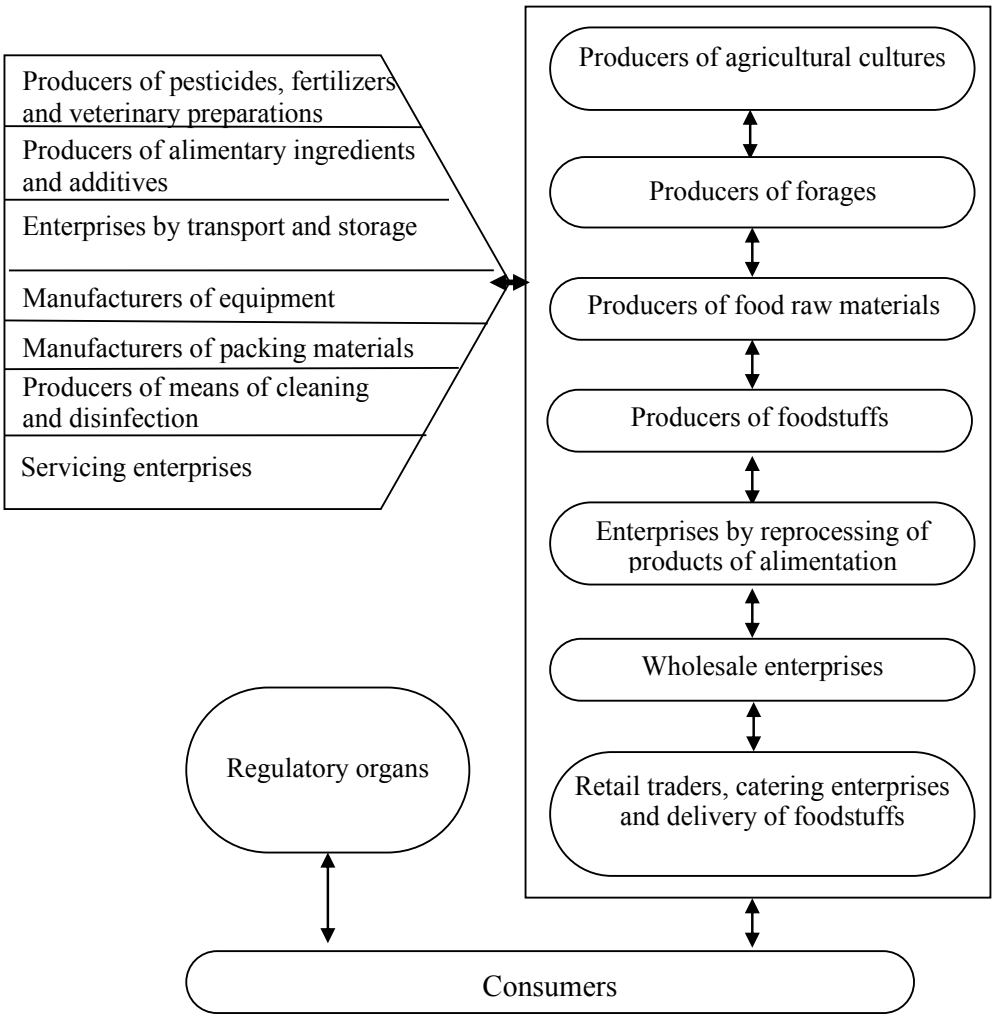

Figure 1 Order of information exchange for raw food.

The auxiliary standard **ISO 22004:2014 (2014)** identifies the objects of control as follows: a) ingredients of foodstuffs, alimentary additives, drinking water, etc., b) processes of treating water, c) state of equipment, condition of working surfaces, packing and other materials, d) compliance with the established requirements of qualification of testing and measuring laboratories and their work procedures, e) observance of norms of personal hygiene and industrial sanitation, f) procedures for storage, transport and distribution of produced foods.

To guarantee observance of the norms established by this document, European persons of the economy have developed several documents regulating the procedures of work for producing safe foods, e.g. the national standards **DS 3027 (2002)** (Denmark standard), **Netherlands specification (2002)**, **BRC (2019)** and **IFC (2019)**.

However, achieving the safety of a certain foodstuff only is not enough in modern conditions because the raw materials and ingredients used are potential sources of hazards, for example, pollution by bacteria, the existence of admixtures of pesticides and heavy metals, and so on. Therefore, the obligatory norm of standard **ISO 22000:2018 (2018)** is the continuous informing of all persons in the food chain (Figure 1).

The typical order of work in assuring the proper quality of manufacture normalizes the international standard **ISO 9001:2015 (2015)**. The ideas at the core of such a document may be classified into four groups: a) planning of quality: establishing the purposes and indices of quality, as well as norms of use of the management system, b) quality management: identification and observance of established norms of work, quality control and correction of non-proper actions, c) assuring quality: works convincing interested parties in the correspondence of objects of control to established norms, d) control and bettering of quality: carrying out work to increase the effectiveness of the enterprise's operation.

At the same time, the quantity of waste formed in the world annually has reached 1.3 billion tons and is estimated to increase to 2.2 billion tons in 2025 **(WHO, 2018)**, so optimizing the procedures of this activity will not solve the problem of assuring healthy conditions of life. Hence here becomes more insistent on the problem of utilization of harmful waste and emissions in the atmosphere, rivers, and seas, because wind, animals, and birds do not know state borders. So, there arose the crucial problem of minimizing the quantity of waste and eliminating sequences of negative human influence on the state of the environment, critical for guaranteeing the health of living and future generations.

We have identified the basic principles of a system of ecological management as a constituent part of a general system of management. They are successfully used in the training of quality management specialists **(Bal-Prylypko et al., 2017)**: a) principle of stable progress: satisfying the needs of humankind realized without the threat of limiting the capability of future generations to use natural resources, b) principle of raising the environmental purity of the enterprise: introduction of measures that assist in decreasing of the level of the negative influence of the person of the economy on the state of the surrounding nature (air, water, and soils) in conditions of the simultaneous raising of the economic and social effects of manufacture.

Karamanos (2001) and **Mitchell (2003)** believed that the components of an environmental management system are: a) consciousness that humankind is an inseparable part of nature and the state of its being depends completely on the state of the environment, b) consciousness of the possibility of health being conditional on the adequate state of natural systems only, c) consciousness of the scantiness of the natural and resource potential of the planet, d) the voluntary limitation of using natural resources up to a level that would not lead to irreversible changes in the state of nature, e) replacing existing goods manufacturing technologies with ecologically compatible ones, f) limitation (if possible) of the global population and the negative influence of its activities on the state of the environment at local, regional and global levels.

People's anxiety about the crucial worsening of global environmental conditions has also found its reflection in the norms of numerous documents and legal instruments devoted to problems of protecting the state of nature in Ukraine **(Law of Ukraine, 1991)**. The result is the development of international standard 14001:2015 'Environmental management systems – Requirements with guidance for use' **(ISO 14001:2015, 2015)**, which establishes the norms for the protection of surrounding nature.

The basic principles of action to reduce environmental damage from industrial waste can be presented in the form of a pyramid **(Kumar and Kumar, 2018)** (Figure 2).

Finally, the staff of food-producing enterprises is live people who are in continual contact with materials, semi-finished products, and finished foodstuffs that contain compounds and substances potentially harmful for their health. At the same time, all work in manufacturing, packing, storage, transporting and distribution involve contact of products with people, which may lead to product contamination and risks to the future health of consumers if hygiene norms are not observed by the personnel of the enterprise. Therefore, a crucial requirement is the observance of the norms of GHP and the creation of healthy and safe working conditions for personnel engaged in manufacture. The norms for organizing such work are found in the international standard **ISO 45001:2018 (2018)**.

Therefore, an enterprise engaged in work with foods must observe at all times the norms of some documents of regulative character, and control of their observance has to be done by specialized departments in its structure. However, the control functions of different kinds of work double in many cases so, to optimize the organizational structure, top management of enterprises has introduced integrated systems of management that comprise all the norms of documents of an administrative character.

We reckon that at a minimum they have to include the norms of four basic international standards, namely **ISO 9001:2015 (2015)**, **ISO 14001:2015 (2015)**, **ISO 22000:2018 (2018),** and **ISO 45001:2018 (2018)**. The layout of the organization of work in the development of such a system is represented in Figure 3 **(Nikolaenko, 2019)**.

Table 2a Succession of work and characteristics of basic norms of standards **ISO 9001:2015**, **ISO 14001:2015**, **ISO 22000:2018** and **ISO 45001: 2018**, used in the development of integrated management system (block 1).

ISO 9001:2015		ISO 14001:2015		ISO 22000:2017		ISO 45001:2018	
Art.	**Part**	**Art.**	**Part**	**Art.**	**Part**	**Art.**	**Part**
0	Scope	0	Scope	0	Introduction	0	Introduction
0.1	General	0.1	General	0.1	General	0.1	Background
0.2	Quality management principles	0.2	Purposes of system of ecological management			0.2	Aim of an OH&S management system
				0.2	FSMS principles		
		0.3	Success factors	—	—	0.3	Success factors
0.3	Process approach	0.4	Plan-Do-Check-Act model	0.3	Process approach	0.4	Plan-Do-Check-Act cycle
						0.5	Content of this document
0.4	Relationship with other management system standards	—	—	0.4	Relationship with other management system standards		
1	Scope	1	Scope	1	Scope	1	Scope
2	Normative references	2	Normative references	2	Normative references	2	Normative references
3	Terms and definitions	3	Terms and definitions	3	Terms and definitions	3	Terms and definitions
4	Context of the organization	4	Context of the organization	4	Context of the organization	4	Context of the organization
4.1	Understanding the organization and its context	4.1	Understanding the organization and its context	4.1	Understanding the organization and its context	4.1	Understanding the organization and its context
4.2	Understanding the needs and expectations of interested parties	4.2	Understanding the needs and expectations of interested parties	4.2	Understanding the needs and expectations of interested parties	4.2	Understanding the needs and expectations of workers and other interested parties
4.3	Determining the scope of the quality management system	4.3	Determining the scope of the EMS	4.3	Determining the scope of the food management system	4.3	Determining the scope of the OH&S management system
4.4	Quality management system and its processes	4.4	Environmental management system	4.4	Food safety management system	4/4	OH&S management system
5	Leadership	5	Leadership	5	Leadership	5	Leadership and worker participation
5.1	Leadership and commitment	5.1	Leadership and commitment	5.1	Leadership and commitment	5.1	Leadership and commitment
5.2	Policy	5.2	Environmental policy	5.2	Policy	5.2	OH&S policy
5.3	Organizational roles, responsibilities and authorities	5.3	Organizational roles, responsibilities and authorities	5.3	Organizational roles, responsibilities and authorities	5.3	Organizational roles, responsibilities and authorities
	—		—		—	5.4	Consultation and participation of workers
6	Planning	6	Planning	6	Planning	6	Planning
6.1	Actions to address risks and opportunities	6.1	Actions to address risks and opportunities	6.1	Actions to address risks and opportunities	6.1	Actions to address risks and opportunities
6.2	Quality objectives and planning to achieve them	6.2	Environmental objectives and plans to achieve them	6.2	Objectives of the food managements system and plans to achieve them	6.2	OH&S objectives and planning to achieve them
6.3	Planning of changes	6.3	Planning of changes	6.3	Planning of changes		—
7	Support	7	Support	7	Support	7	Support
7.1	Resources	7.1	Resources	7.1	Resources	7.1	Resources
7.2	Competence	7.2	Competence	7.2	Competence	7.2	Competence
7.3	Awareness	7.3	Awareness	7.3	Awareness	7.3	Awareness

Table 2b Succession of work and characteristics of basic norms of standards **ISO 9001:2015**, **ISO 14001:2015**, **ISO 22000:2018** and **ISO 45001:2018**, used in the development of integrated management system (block 2).

ISO 9001:2015		ISO 14001:2015		ISO 22000:2017		ISO 45001:2018	
Art.	**Part**	**Art.**	**Part**	**Art.**	**Part**	**Art.**	**Part**
7.4	Communication	7.4	Communication	7.4	Communication	7.4	Communication
7.5	Documented information	7.5	Documented information	7.5	Documented information	7.5	Documented information
8	Operation	8	Operation	8	Operation	8	Operation
8.1	Operational planning and control	8.1	Operational planning and control	8.1	Operational planning and control	8.1	Operational planning and control
8.2	Requirements for products and services	—	—	—	—		
8.3	Design and development of products and services	—	—	8.2	Prerequisite programs		
8.4	Control of externally provided processes, products and services	—	—	8.3	Traceability system		
8.5	Production and services provisions	—	—				
8.6	Release of products and services	—	—				
8.7	Control of nonconforming outputs						
	_—	8.2	Emergence preparedness and response	8.4	Emergence preparedness and response	8.4	Emergence preparedness and response
	—		—	8.5	Hazard control		
	—		—	8.6	Updating the information specifying the PRPs and the hazard control plan		—
	—		—	8.7	Control of monitoring and measuring		—
	—		—	8.8	Verification related to PRPs and the hazard control plan		—
	—		—	8.9	Control of product and process nonconformities		—
9	Performance evaluation	9	Performance evaluation	9	Performance evaluation	9	Performance evaluation
9.1	Monitoring, measurement, analysis and evaluation	9.1	Monitoring, measurement, analysis and evaluation	9.1	Monitoring, measurement, analysis and evaluation	9.1	Monitoring, measurement, analysis and performance evaluation
9.2	Internal audit	9.2	Internal audit	9.2	Internal audit	9.2	Internal audit
9.3	Management review	9.3	Management review	9.3	Management review	9.3	Management review
10	Improvement	10	Improvement	10	Improvement	10	Improvement
10.1	General	10.1	General	—	—	10.1	General
10.2	Nonconformity and corrective action	10.2	Non-conformity and corrective action	10.1	Non-conformity and corrective action	10.2	Incident, conformity and corrective action
10.3	Continual improvement	10.3	Continual improvement	10.2	Continual improvement	10.3	Continual improvement
—	—	—		10.3	Update of the foods safety management system		—

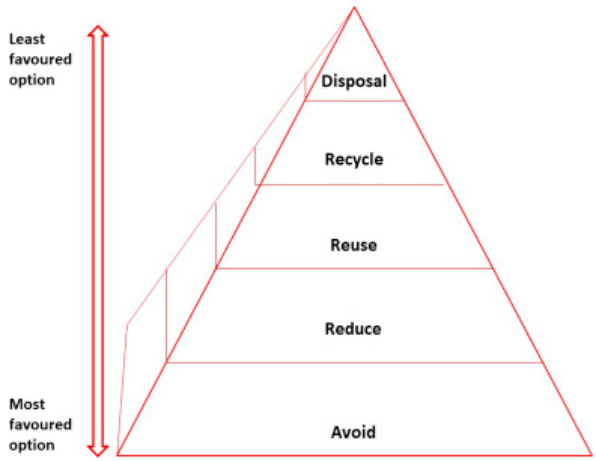

Figure 2 Waste management practice.

This system is also used in the educational process of our university.

The universality of methodology and most of the norms of ISO 9001 were used as a basis for developing system permits to simplify the task of integrating the activities of meat processing enterprises into one management system by the addition of the above standards.

The system offered by us successfully works in the advanced enterprises of Ukraine. We plan to patent it in the future. The succession of works in the development of integrated management systems is shown in Table 2a and Table 2b (blocks 1 and 2) **(Nikolaenko, 2019)**.

The posting shown in Table 2a and Table 2b may be recommended as a typical one in the development of complex integrated quality management systems in meat processing enterprises. The distinctive feature of its chapters is that those have to be taken in the order given in the table.

In Ukraine, the basic document in this area of meat production is the Law of Ukraine 'On Basic Principles and Requirements for Food Safety and Quality' **(Law of Ukraine, 1998)**. However, the norms for production organization established by it have a fragmentary character and cannot be recommended as a basis on which the corresponding products can be designed and put into operation **(Kyryliuk and Kyryliuk, 2017)**. In our opinion, the main reasons are the failure to take into account the provisions of good practices – production, hygiene, distribution, laboratory activities, etc. We eliminate all this in the proposed integrated system (Table 2a and Table 2b). In the proposed variant, the production of meat products is organized in such a way that the probability of occurrence of undesirable situations and a negative impact on the environment is minimized. Today, food legislation in Ukraine is also evolving. The new **Law of Ukraine (2018)** has come into force.

The development was based on the provisions of the Codex Alimentarius Commission, the HACCP principles, and the document **SQF (1995)**. Of greatest practical interest in Ukraine for producers of meat and meat products is Module 11: Food Safety Fundamentals – Good Manufacturing Practices for Processing of Food Products – GFSI El, Ell, Elll, ElV, and L **(SQF, 2017)**. This is especially important for products of animal origin and their processing with a short shelf life and sale.

There is continuous improvement of food production technologies **(Medina et al., 2019)** and the introduction of new product recipes, including those using non-traditional raw materials **(Tavdidishvili et al., 2020)**. All this also requires a comprehensive approach to risk management in the field of meat production.

Microstructural studies of ready-to-cook chopped meat products allow identification of their components, the establishment of the different properties of various tissue and cellular structures, and control of the manufactured articles **(Paska et al., 2019)**. Minced beef as the object of research was modified, with 5%, 10%, or 15% of the meat part replaced with lupin flour and 0.5% with elecampane root powder added as an aromatic raw material. It has been shown that histological studies, with the PAS reaction used, help determine the meat and plant content in the ready-to-cook meat developed, and that hematoxylin and eosin can help determine the functional ingredient content. With this in mind, we conclude that the traceability of meat products is important at all stages, which will ensure an integrated quality system.

Regardless of the size and scope of the enterprise, when developing an integrated management system (IMS) in accordance to the requirements of two or more standards it should be developed a single comprehensive documentation, policy, documented information (procedures, forms of records), processes and management assessment that would create a universal management system for individual objects (quality, food safety, ecology, occupation health and safety, etc.) guided by the general approach of ISO standards to management systems.

Guided by Table 2a and Table 2b, we developed an algorithm for the implementation of IMS, taking into account the requirements of the four standards for management systems in the meat processing plant, presented in Figure 3.

To develop and implement the IMS, a team of specialists from each field (business process management, food safety, ecology, and labour protection) should be established, and a person will be appointed to manage and coordinate the team's activities and be responsible for the development and operation of the overall management system. That is, the IMS managing group should consist of 4 – 5 people. Usually, selected individuals must have appropriate qualifications in their field, understand the processes and manage them, and have sufficient knowledge of the requirements of individual standards. The IMS team leader must have the competence, knowledge, and ability to apply general approaches and requirements to management systems according to selected standards and rules and conditions of integration of management systems, as well as have leadership qualities for overall management and coordination of the IMS group. In the future, it is the IMS group and its head who are responsible for implementing the algorithm for implementing IMS in production.

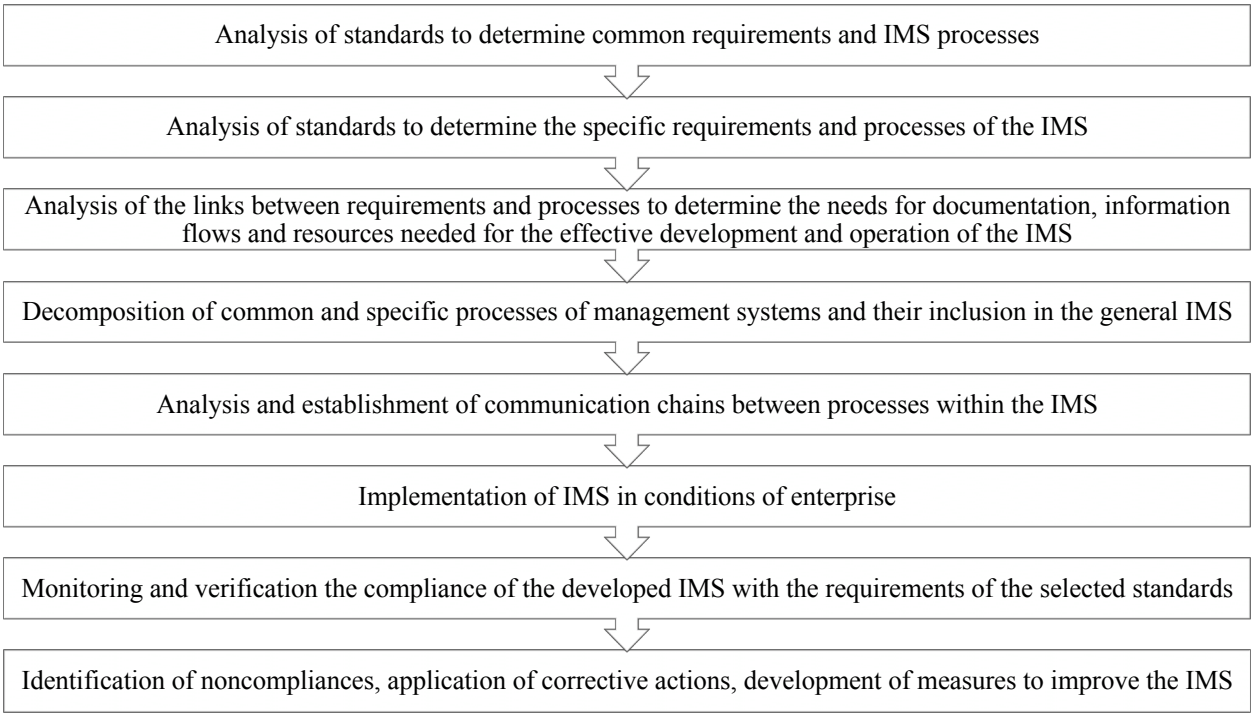

Figure 3 Algorithm of the introduction of IMS in the conditions of the meat-processing enterprises.

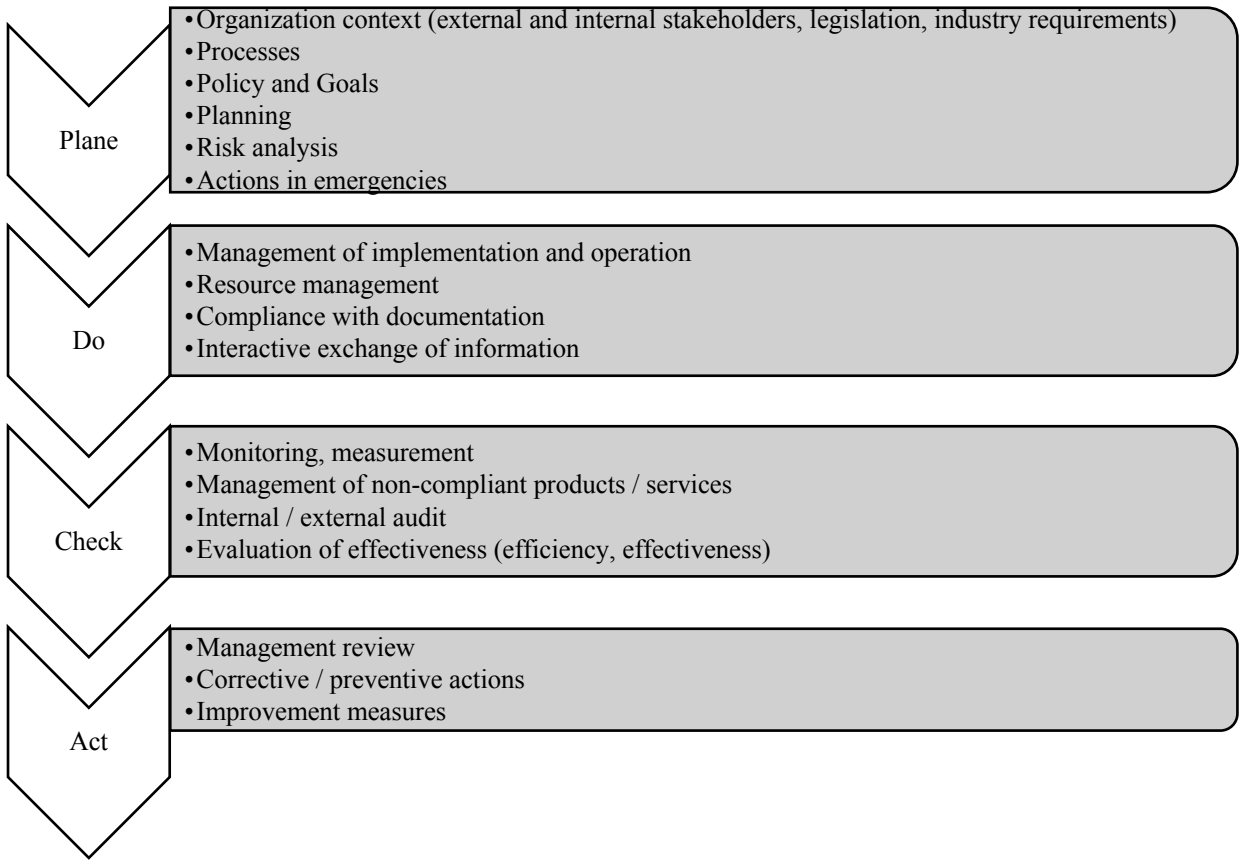

Figure 4 General requirements for IMS, taking into account the cycle Plan-Do-Check-Act.

Applying **PAC 99 Integrated Management (2016)** we have identified common elements of the IMS, taking into account the structure and requirements of the standards **ISO 9001:2015 (2015)**, **ISO 14001:2015 (2015)**, **ISO 22000:2018 (2018)**, **ISO 45001:2018 (2018)**, and the cycle Plan-Do-Check-Act (Figure 4).

Among the common requirements is the requirement to establish the context of the organization, i. e. to identify internal and external stakeholders in the activities of the organization, which either affect the organization or are affected by its activities. Where necessary, the limits of the application of the IMS are agreed with the stakeholders, unless otherwise specified in the contractual obligations or the legislation of the country.

The IMS should not only include external standards and specifications but should also be guided by industry or internal instructions and documentation, provided they are available and acceptable.

Next, the scope of the IMS should be defined, which includes standards, structural units of the enterprise, production sites, products/services, etc.

After defining the scope of the IMS, the management, core, and support processes required for the development, implementation, and operation of the IMS should be identified and the necessary processes should be available in the organization. When determining the processes involved in the IMS, it should be borne in mind that all processes necessary for the proper functioning of the system should be involved, including accounting, marketing, personnel management, etc., because without the inclusion of these processes it is impossible to produce/sell products, services.

In addition to determining the necessary processes, it is necessary to establish their sequence, interaction, and relationship, to establish the criteria and methods necessary to ensure the effectiveness of processes within the IMS.

For each process, it is necessary to develop maps of processes, the main purpose of which is to illustrate the technology of the process and reflect the movement of flows from inputs to outputs between departments and structural units of the enterprise. Exits from one process should be entirely inputs to other processes, and the reflection of inputs and outputs of all involved processes in the enterprise should reflect the relationship and responsibility for the compliance of the final product and activities to the requirements of stakeholders. Process maps should reflect the process with the degree of detail that is necessary to obtain reliable, reproducible, and acceptable process results. The process map is visualized in any form, but there are a number of mandatory elements that should be displayed, among them: name of the process; process operations; resources necessary for the proper course of the process (material, technical, human, information, etc.); competence and qualification of staff; special conditions or parameters of the process; documentation containing requirements for the process product; methods of process monitoring; reporting documentation on the implementation of the process; methods of checking the effectiveness of the process.

As a rule, the graphic and text form of process maps is most often used, which combines a block diagram of the process and a text description of it.

In the future, guided by the requirements of the selected standards for IMS and process maps, the structure of IMS documentation is created. If you define the general documentation that should be within the IMS, it is: IMS policy; objectives taking into account the scope of management of IMS standards; general organizational rules of the enterprise taking into account the requirements of the legislation, stakeholders and the accepted corporate culture; rules of daily routine and remuneration; IMS Guide, which describes the guidelines, processes, and documentation structure of the management system; procedures and documented information as required by relevant standards, specifications used and included in the IMS; documentation required for proper planning, operation, and monitoring of the effectiveness of IMS processes.

The company's policy should include all standards of management systems involved in the IMS, as well as clearly reflect the scope of the system. Objectives should be measurable, achievable, relevant, and include all aspects of management: quality, food safety, environmental and occupational health, and safety. In addition, the goals in different aspects of the IMS should not contradict each other and be clearly understood.

The main document of the IMS, which describes the established management system, is the IMS Guide.

The indicative structure of the IMS Guide is presented below:

1 Integrated management system
- 1.1 Terms
- 1.2 Scope
- 1.3 The organizational structure of IMS
- 1.4 Description of process interaction

2 Regulatory references

3 Terms and definitions

4 Requirements for documented information
- 4.1 General regulations
- 4.2 IMS Guide
- 4.3 Document management
- Records management

5.1 The context of the organization
- 5.1 Defining the context of the organization
- 5.2 Identifying the needs of stakeholders

6 Leadership
- 6.1 Leadership and commitment and leadership responsibilities
- 6.2 Consumer orientation
- 6.3 IMS policy formation
- 6.4 Responsibility and authority

7 Planning
- 7.1 Quality management system
  - 7.1.1 Risks and opportunities
  - 7.1.2 Management measures
- 7.2 Environmental management system
  - 7.2.1 Environmental aspects
  - 7.2.2 Management measures
- 7.3 Food safety management system
  - 7.3.1 Programs are prerequisites
  - 7.3.2 Dangerous factors
  - 7.3.3 Prerequisite programs
  - 7.3.4 Critical control points
  - 7.3.5 Management measures

7.4 Occupational safety and health management system
7.4.1 Hazard identification and risk assessment
7.4.2 Management measures
8 Provision
8.1 Provision of resources
8.2 Human resources, competence and awareness
8.3 Infrastructure
8.4 Functional process environment
8.5 Resources for monitoring and measurement
8.5.1 General regulations
8.5.2 Traceability of measurements
8.6 Knowledge management
8.7 Interactive information exchange
9 Production activity
9.1 Production process planning
9.2 Requirements for products and services
9.3 Definition, analysis and changes in requirements for products and services
9.4 Design and development of new products and services
9.5 Management of processes, products and services supplied by external suppliers
9.5.1 Type and degree of control
9.5.2 Informing suppliers
9.6 Production
9.6.1 Production control
9.6.2 Identification and traceability
9.6.3 Property owned by consumers or external suppliers
9.6.4 Storage
9.7 Supply of products and services
9.8 Change management in production
9.9 Management of inappropriate products and services
9.10 Planning and management of IMS work
9.11 Readiness for emergency stock
10 Evaluation of performance
10.1 General regulations
10.2 Satisfying the needs of consumers
10.3 Analysis and evaluation
10.4 Investigation of events / complaints
10.5 Internal audits
10.6 Management analysis
11 Improvements
11.1 General regulations
11.2 Corrections and corrective actions

After the implementation of the IMS, process monitoring procedures should be provided to obtain objective evidence of the effectiveness of the management system taking into account the established and defined requirements, as well as validation and verification of processes, documentation and overall IMS to assess the effectiveness of the IMS and policy compliance standards and stakeholders.

As a rule, conformity assessment is based on the results of internal and external audits. The overall evaluation of the IMS and the development of measures to improve the management system is based on the results of management analysis. After the analysis, the management updates the policy, goals, resource planning, and the next cycle of the IMS.

CONCLUSION

The principles of a systematic approach to the development of an integrated management system for quality and product safety of meat processing enterprises in Ukraine were formulated and put into practice. This made it possible to develop a plan for risk control and critical control for meat processing plants in Ukraine, taking into account best practices across the world. An integrated system for managing product quality and safety for meat processing enterprises operating in conditions of minimal environmental damage has been developed.

In conclusion, it should be noted that the introduction of IMS in food enterprises in general and meat processing, in particular, will provide a number of advantages:

1 prevent conflicts between management systems that are already in place or planned to be implemented;

2 reduce the level of duplication of documentation, powers, responsibilities and the general level of bureaucratization of the company's management;

3 due to the coherence of the IMS processes, the efficiency and effectiveness of the company's activities are increased and the general coherence of the actions of all structural subdivisions, both production, and non-production, are achieved;

4 a company that implements IMS receives a number of benefits that are aimed at optimizing the external and internal environment (context), and therefore focus on customer and stakeholder satisfaction while meeting the requirements of international standards and best international practices.

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THE INFLUENCE OF HONEY ENRICHMENT WITH BEE POLLEN OR BEE BREAD ON THE CONTENT OF SELECTED MINERAL COMPONENTS IN MULTIFLORAL HONEY

Celina Habryka, Robert Socha, Lesław Juszczak

ABSTRACT
Bee products, such as honey, pollen, and bee bread, are an excellent source of bioactive ingredients, including minerals, having a health-supporting effect. However, due to the specific sensory properties of bee pollen and bee bread, the best way to include them in a diet is to add them to honey. Therefore, the aim of this paper was to evaluate the influence of the added bee pollen or bee bread on selected minerals content in multifloral honey. The mineral content was analyzed using absorption atomic spectrometry (FAAS) with prior dry mineralization. On the basis of obtained results, it was found that the addition of bee pollen or bee bread to honey significantly influences the content of selected macro- and microelements, excluding sodium. The greatest increase in mineral content was observed for magnesium, iron, and zinc. Enrichment of honey with the highest dose of bee pollen or bee bread resulted in an over 20-fold increase in the Mg and Fe content, and an over 14-fold increase in the Zn content. Honey enriched with the maximum addition of bee pollen was characterized by a higher content of K, Ca, Mg, Fe, and Cu compared to honey with bee bread. Due to a fact that both bee pollen and bee bread are good sources of minerals, their addition to honey significantly increases its ability to cover daily demand for macro- and microelements.

Keywords: honey; bee pollen; bee bread; mineral composition

INTRODUCTION
Honey is a naturally sweet substance produced by honey bees *Apis mellifera* from plant nectar, secretions of live parts of plants or insect excretions. Honey has been valued for centuries, in different countries and cultures, due to a diversified range of its preventive and health-supporting properties **(Bogdanov et al., 2008; Socha, Habryka and Juszczak, 2016; Socha, Habryka and Juszczak, 2018)**. Consumption of natural honey contributes to improved immunity and enriches diet in many valuable nutrients and bioactive substances. It is an excellent form of easily digestible sugars, vitamins, organic acids, and many other biologically active substances **(Bogdanov et al., 2008; Wesołowska and Dżugan, 2017)**. Due to its highly diversified chemical composition, it is used as a treatment supporting agent **(Bogdanov et al., 2008)**. Apart from direct consumption, it is used to sweeten food and drinks, baking, preserving food, and preparing of mead **(Dżugan, Ruszel and Tomczyk, 2018)**.

Honey contains mineral ingredients deriving mainly from plant juice creating nectar, or from honeydew. The mineral ingredients content in honey depends on its variety and environmental conditions **(Kačániová et al., 2009; Grembecka and Szefer, 2013)**. Differences in contents of bioelements between varieties, or even within the same variety of honey result from their varying content in obtained plant material **(Pohl, Sergiel and Stecka, 2009; Kędzierska-Matysek et al., 2013; Dżugan et al., 2017)**. Levels of these compounds are the lowest in light nectar honey, while they are the highest in honeydew honey **(Gonzalez-Miret et al., 2005)**. Potassium is the main mineral ingredient found in honey, and its content corresponds to half of the total mineral content. Magnesium is found at a relatively stable level in honey, while the calcium content can vary significantly, depending on the honey origin **(Dżugan et al., 2017)**. Phosphorus, iron, manganese, silicon, nickel, and sulfur are found in smaller quantities, while trace elements include copper, barium, cobalt, zinc, tin, palladium, aluminum, tungsten, chromium, titanium, molybdenum, vanadium, cadmium, and others **(Solayman et al., 2016)**. Differences in macro- and microelements contents within the same honey variety results from the biodiversity of material, which contributes to a significant diversification of its mineral composition **(Gonzalez-Miret et al., 2005; Kędzierska-Matysek et al., 2013)**. The number of mineral ingredients found in honey is correlated with species of plants from which the material is harvested. The botanical origin, in turn, is closely correlated with the location of an apiary, because soil composition and climatic conditions also determine the presence of metals in

melliferous plants. Environmental pollution and other anthropogenic processes should also be considered as an additional source of metals in honey, including Cu, Fe, and Zn **(Kačániová et al., 2009; Pohl, Sergiel and Stecka, 2009; Roman and Popiela, 2011; Solayman et al., 2016)**.

Apart from honey, bee products also include pollen, bee bread, propolis, royal jelly, bee venom, and wax. Pollen, depending on plants visited by bees, forms pollen loads of a color characteristic for a given plant species. Its composition is varied and depends on the origin and on weather conditions prevailing during anther forming and maturing **(Gabriele et al., 2015; Kędzia and Hołderna-Kędzia, 2016)**. Bee pollen is a diversified plant product rich in biologically active ingredients. Over 200 active substances were found in it **(Gabriele et al., 2015; Kieliszek et al., 2018)**. Bee pollen is also rich in valuable bioelements essential for human health. They include macroelements such as phosphorus, potassium, magnesium, and calcium, microelements such as iron, manganese, zinc, and trace elements like copper, cobalt, and nickel **(Kędzia and Hołderna-Kędzia, 2016)**.

The bee bread is formed from plant pollen preserved by bees. Bees place flower pollen loads in honeycomb cells, wetting it with the secretion of their salivary glands and honey. Then bees pack it tightly in the cells to secure it against the access of air **(Kieliszek et al., 2018; Bakour et al., 2019)**. In anaerobic conditions, this mixture of pollen, honey, and bee saliva is fermented by *Lactobacillus* bacteria, which contribute to the formation of antibacterial peptides, hydrogen peroxide, and organic acids. Enzymes found in bee saliva and bacteria themselves contribute to differences in the chemical composition of fresh pollen loads and bee bread **(Kieliszek et al., 2018; Bakour et al., 2019)**. When compared to pollen, bee bread is characterized by higher nutritional value, better digestibility, and richer chemical composition **(Socha, Habryka and Juszczak, 2016)**.

The literature review indicates that bee products such as bee pollen and bee bread are an excellent source of bioactive ingredients, and their addition to the diet may significantly enrich it with bioactive substances, including minerals, having health-supporting effects. Due to the specific sensory properties of bee pollen and bee bread, apparently, the best way to include them in a diet is to add them to honey **(Kňazovická et al., 2011; Juszczak et al., 2015)**.

Therefore, the aim of this paper was to evaluate the influence of the added bee pollen or bee bread on selected minerals content in multifloral honey.

Scientific hypothesis

Enrichment of honey with bee pollen or propolis at a sensoric acceptable level causes a significant increase in the content of mineral components.

MATERIAL AND METHODOLOGY

Materials

The multiflower honey (District Beekeeping Cooperative "Pszczelarz", Krakow, Poland) and the bee pollen and bee bread (Biopharmaceutical Laboratory „Aria", Krakow, Poland) were used as experimental materials. Based on the preliminary sensory assessment, honey was enriched with bee pollen or bee bread in a quantity ranging from 5% to 25%. The use of the maximum pollen addition to honey, at a level of 25%, was supported by conducted preliminary sensory evaluation. The increasing levels of pollen or bee bread in honey changed the perception of its color, smell, texture, and palatability.

Methods

Determination of the chosen mineral components content was made by atomic absorption spectrometry (ASA) in accordance with the requirements of the Polish Standard **(PN-EN 14082, 2004)**. Mineralization of the tested sample was carried out at 600 °C in a muffle furnace (SNOL 8.2/1100, Lithuania) for 12 h. After mineralization, the ash content was calculated. The mineralized samples were dissolved in hot 6.0M hydrochloric acid and then in 0.5 M nitric acid. The chosen mineral components in honey were determined with an Avanta Sigma atomic absorption spectrometer (GBC, Australia), flame technique (FAAS), using acetylene/air or, for calcium, acetylene/nitrogen monoxide). The levels of macroelements including calcium, magnesium, sodium, and potassium as well as microelements: iron, zinc, copper, and manganese were determined. The quantitative analysis was carried out on a base of calibration curves made for standard solutions (Merck, Germany). The work parameters of the spectrometer are given in Table 1.

Table 1 Spectrometer parameters used in the mineral composition analysis.

Element	Wavelength (nm)	Working range (μg.ml^{-1})	Equation of the calibration graph	R^2
K	769.9	2.0 – 12.0	y = 8.947x + 0.017	0.9998
Na	589.6	0.2 – 1.5	y = 6.320x - 0.023	0.9983
Ca	422.7	1.0 – 4.0	y = 42.421x - 0.343	0.9905
Mg	202.6	0.5 – 1.5	y = 0.799x + 0.025	0.9872
Fe	248.3	0.2 – 0.4	y = 23.894x – 0.422	0.9948
Zn	213.9	1.0 – 0.8	y = 3.925x + 0.071	0.9981
Cu	324.7	0.1 – 1.5	y = 12.368x + 0.066	0.9995
Mn	279.8	0.2 – 0.4	y = 8.988x + 0.028	0.9973

Statistical analysis

The analyses were made in triplicate, and the results were expressed as mean values ± standard deviations. In order to determine the significant differences between the means, the data were treated by one-way analysis of variance, and the Fisher test at a significance level α = 0.05 was calculated.

In order to assess the impact of both the type and amount of additive level (i.e., bee pollen or bee bread) to honey a two-way analysis of variance was performed. The values of Pearson's linear correlation coefficients between ash content and the content of individual mineral components were calculated, and their significance was verified by the Student's t-test at the significance level of 0.05. Calculations were performed with statistical software package Statistica 11.0 (StatSoft Inc., USA).

RESULTS AND DISCUSSION

The physical and chemical properties and nutritional value of honey are significantly influenced by its ash content, which also reflects the content of total and individual minerals. The ash content is a parameter depending on botanical origin, and it is also correlated with honey color **(Gonzalez-Miret et al., 2005; Pohl, Sergiel and Stecka, 2009)**. The ash content in the studied multifloral honey and in honey enriched with bee pollen or bee bread is shown in Figure 1. The determined ash content was 0.19 g per 100 g of pure multifloral honey.

The obtained results are within the extensive range specified for Polish multifloral nectar honeys **(Kędzierska-Matysek et al., 2013)**. The increasing addition of pollen or bee bread contributed to a significant (p <0.05) proportional increase in the honey ash content.

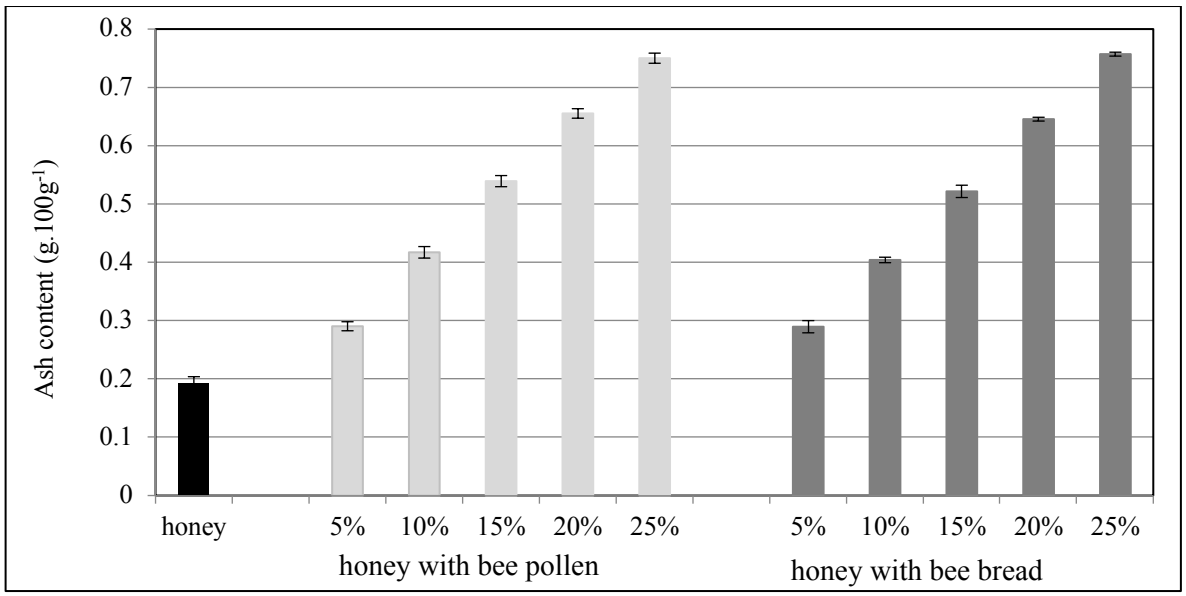

Figure 1 Ash content of multifloral honey and samples enriched with pollen or bee bread.

Table 1 Macroelements content in multifloral honey and samples enriched with pollen or beebread.

Type of ingredient	Amount of ingredient (%)	K (mg.100g^{-1} ±SD)	Na (mg.100g^{-1} ±SD)	Ca (mg.100g^{-1} ±SD)	Mg (mg.100g^{-1} ±SD)
	0	271.4 ±3.8^{j}	8.1 ±0.1^{a}	13.9 ±0.4^{j}	2.3 ±0.2^{j}
	5	299.4 ±6.2^{i}	8.3 ±0.4^{a}	23.9 ±2.5^{h}	11.5 ±0.5^{i}
honey	10	372.7 ±4.8^{g}	7.8 ±0.9^{a}	31.4 ±1.6^{g}	21.4 ±0.4^{g}
bee pollen	15	486.4 ±5.1^{e}	8.1 ±0.7^{a}	45.4 ±0.9^{d}	33.4 ±0.3^{e}
	20	548.1 ±10.8^{c}	8.1 ±0.5^{a}	53.0 ±1.2^{b}	41.2 ±0.7^{c}
	25	639.3 ±9.5^{a}	8.1 ±0.9^{a}	67.8 ±1.0^{a}	50.7 ±0.3^{a}
	5	350.8 ±3.0^{h}	7.6 ±0.7^{a}	21.5 ±0.4^{i}	13.1 ±0.4^{h}
	10	364.6 ±2.8^{g}	8.5 ±0.7^{a}	31.4 ±1.7^{g}	22.0 ±0.1^{g}
bee bread	15	419.5 ±7.2^{f}	8.0 ±0.4^{a}	34.2 ±0.2^{f}	29.4 ±0.2^{f}
	20	524.6 ±1.8^{d}	7.5 ±0.5^{a}	40.1 ±1.4^{e}	35.8 ±0.2^{d}
	25	617.8 ±3.3^{b}	8.0 ±0.2^{a}	50.1 ±2.6^{c}	46.2 ±0.5^{b}
Two-way ANOVA - p					
Factor I (Type)		p <0.05	p = 0.487	p <0.05	p <0.05
Factor II (Amount)		p <0.05	p = 0.936	p <0.05	p <0.05
Factor I x Factor II		p <0.05	p = 0.398	p <0.05	p <0.05

Note: Mean values assigned with the same letters in particular columns are non-significant at the 0.05 level of confidence.

Table 1 list average contents of macroelements in multifloral honey and in honeys enriched with bee pollen or bee bread. In the plain multifloral honey, potassium was an element found at the highest level, and its content corresponded to over 90% of the total minerals determined. Its average content amounted to 271.4 mg per 100 g (Table 1), and this confirms earlier results for multifloral honeys **(Grembecka and Szefer, 2013; Kędzierska-Matysek et al., 2013; Juszczak et al., 2018)**. The addition of pollen to honey significantly (p <0.05) increased its potassium levels. The greatest increase in potassium content, to 639.3 mg.100g^{-1} was noted for 25% concentration of bee pollen in honey.

This observation confirms results obtained by **Juszczak et al. (2018)** for the commercial samples of honey with pollen added, who determined its potassium content at a level of 494 mg to 651 mg per 100 g. A significant (p <0.05) increase in potassium levels in honey enriched with pollen results from the high content of that ingredient in pollen. According to **Kędzia and Hołderna-Kędzia (2016)**, potassium content in pollen ranges from 284.3 mg to 2000 mg per 100 g. The potassium content in honey enriched with bee bread increases in a similar way, where a 5% addition already increased the content of that element to 350.8 mg.100g^{-1} and a 25% addition increases that content to the level of 617.8 mg.100g^{-1} (Table 1). Much higher average potassium content in commercial samples of honey enriched with bee bread was observed by **Juszczak et al. (2018)**, and this resulted from a significantly higher addition of bee bread. The conducted two-way analysis of variance has shown that the potassium content in enriched honey significantly (p <0.05) depends both on the type and the level of the added ingredient. Furthermore, a significant positive linear correlation (r = 0.9823) was found between ash and potassium content. A significant increase in potassium content following the addition of bee products to honey significantly influences its nutritional value. While 100 g of multifloral honey covers ca. 13% of the daily potassium demand, products enriched with pollen or bee bread cover over 39% of that demand.

The sodium content in the analyzed multifloral honey amounted to 8.1 mg.100g^{-1}, representing ca. 2.7% of the total determined mineral ingredients (Table 1), and this confirms previous observations for Polish multifloral honey **(Kędzierska-Matysek et al., 2013)**. The addition of bee products did not result in a significant (p >0.05) change in contents of this element, and this observation was confirmed by the two-way analysis of variance (Table 1). Studies conducted by **Grembecka and Szefer (2013)** show that the bee pollen sodium content amounts to 5.86 mg.100g^{-1}, while **Bakour et al. (2019)** determined the sodium content in bee bread at a level of 14.2 mg.100g^{-1}.

Thus, the literature data indicates that the sodium content of bee pollen and bee bread is similar to its content in honey, therefore, their addition does not significantly (p >0.05) influence the content of that macroelement.

The calcium content in the analyzed multifloral honey amounted to 13.9 mg.100g^{-1}, representing ca. 4.7% of the total determined mineral ingredients (Table 1), and is within an extensive range reported for Polish multifloral honey **(Grembecka and Szefer, 2013, Kędzierska-Matysek et al., 2013, Juszczak et al., 2018)**.

Increasing addition of bee pollen to honey increased the calcium content from 23.9 mg to 67.8 mg per 100 g of the product, while enrichment with bee bread increased the content of this element from 21.5 mg to 50.1 mg.100g^{-1} (Table 1). The literature data indicate that the calcium content in pollen is much higher than in multifloral honey **(Grembecka and Szefer, 2013)**, hence its significant (p <0.05) increase after the enrichment. The conducted two-way analysis of variance has shown that the calcium content in enriched honey significantly (p <0.05) depends both on the type and the level of the added substance. Furthermore, a significant positive linear correlation (r = 0.9625) was found between ash and calcium content. Although honey is not a good source of calcium because at the determined content per 100 g (Table 1) it covers less than 2% of daily demand for this element, yet when honey is enriched with pollen or bee bread, the coverage of the daily calcium demand rises to ca. 6% to 8% per 100 g of the product.

Table 2 Microelements content in multifloral honey and samples enriched with pollen or bee bread.

Type of ingredient	Amount of ingredient (%)	Fe (mg.100g^{-1} ±SD)	Zn (mg.100g^{-1} ±SD)	Cu (mg.100g^{-1} ±SD)	Mn (mg.100g^{-1} ±SD)
honey	0	0.34 ±0.03^{k}	0.19 ±0.00^{i}	0.10 ±0.00^{i}	1.20 ±0.01^{k}
bee pollen	5	1.29 ±0.05^{j}	0.53 ±0.01^{h}	0.12 ±0.01^{h}	1.29 ±0.02^{j}
	10	3.90 ±0.09^{f}	0.95 ±0.00^{f}	0.16 ±0.01^{g}	1.48 ±0.01^{i}
	15	4.55 ±0.17^{e}	1.67 ±0.01^{d}	0.28 ±0.02^{de}	1.91 ±0.00^{h}
	20	6.67 ±0.04^{c}	2.25 ±0.05^{c}	0.41 ±0.02^{c}	2.07 ±0.01^{g}
	25	9.67 ±0.17^{a}	2.73 ±0.02^{b}	0.57 ±0.04^{a}	2.35 ±0.01^{e}
bee bread	5	2.59 ±0.07^{i}	0.87 ±0.00^{g}	0.15 ±0.00^{g}	2.15 ±0.01^{f}
	10	3.73 ±0.03^{g}	1.50 ±0.00^{e}	0.18 ±0.01^{f}	2.89 ±0.02^{d}
	15	3.38 ±0.03^{h}	2.27 ±0.05^{c}	0.26 ±0.02^{e}	3.61 ±0.02^{c}
	20	5.01 ±0.24^{d}	2.74 ±0.01^{b}	0.30 ±0.02^{d}	4.21 ±0.01^{b}
	25	7.26 ±0.19^{b}	3.75 ±0.02^{a}	0.46 ±0.01^{b}	4.92 ±0.04^{a}
Two-way ANOVA – p					
Factor I (Type)		p <0.05	p <0.05	p <0.05	p <0.05
Factor II (Amount)		p <0.05	p <0.05	p <0.05	p <0.05
Factor I x Factor II		p <0.05	p <0.05	p <0.05	p <0.05

Note: Mean values assigned with the same letters in particular columns are non-significant at the 0.05 level of confidence.

In the analyzed multifloral honey, magnesium was at the level of 2.3 mg.100g^{-1} (Table 1), and this corresponds to over 0.74% of the total mineral ingredients determined. The determined magnesium content was within the extensive range reported for Polish multifloral honeys **(Grembecka and Szefer, 2013; Kędzierska-Matysek et al., 2013; Juszczak et al., 2018)**. The addition of bee pollen or bee bread significantly (p <0.05) influences an increase in the magnesium content, and this observation was confirmed by the results of the two-way analysis of variance (Table 1).

As reported by **Grembecka and Szefer (2013)**, floral pollen is richer in magnesium than honey, therefore, its addition contributed to a significant (p <0.05) increase in contents of that element to a level of 50.7 mg.100g^{-1} in the enriched honey. In honey enriched with bee bread content of that element was not much lower (Table 1). Although honey is not a good source of magnesium because at the determined content per 100 g (Table 1) it covers only about 0.6% of daily demand for this element, yet when honey is enriched with pollen or bee bread, the coverage of the daily magnesium demand rises to ca. 12% to 13% per 100 g of the product.

Determinations also included contents of iron, zinc, copper, and manganese in multifloral and enriched honey. The average contents of these microelements are presented in Table 2. Iron is an element that in the studied honey represented 0.11% of all determined mineral compounds, and its content amounts to 0.34 mg.100g^{-1} (Table 2). The obtained result was within the extensive range reported for Polish multifloral honey **(Grembecka and Szefer, 2013, Kędzierska-Matysek et al. 2013)**. The addition of bee pollen to honey at a level of 25% contributed to the significantly (p <0.05) increase in the iron content to the level of 9.67 mg.100g^{-1}, while the addition of the bee bread increased the iron content to the value of 7.26 mg.100g^{-1}. Slightly lower values for the iron content in samples of commercial honey enriched with pollen or bee bread were reported by **Juszczak et al. (2018)**. According to **Grembecka and Szefer (2013)**, bee pollen is a rich source of iron, and its content ranges from 3.26 to 3.96 mg.100g^{-1}. For this reason, honey enrichment with these bee products significantly (p <0.05) contributes to the increase in the level of that microelement. The conducted two-way analysis of variance has shown that the iron content in enriched honey significantly depends both on the type and the level of the added substance. Furthermore, a significant positive linear correlation (r = 0.9471) was found between ash and iron content. A significant increase in iron content following the addition of bee products to honey significantly influences its nutritional value. While 100 g of multifloral honey covers ca. 2.5% of the daily iron demand, enriched honey cover as much as 69% of that demand for bee pollen and ca. 52% for bee bread.

In the analyzed honey, the zinc content was at the level of 0.19 mg.100g^{-1} (Table 2), and this corresponds to over 0.06% of the total mineral ingredients determined. The obtained values are consistent with data reported by **Grembecka and Szefer (2013)** for Polish honey, and by **Kačániová et al. (2009)** for Slovak honey. Other report indicate a slightly higher Zn content in Polish multifloral honey **(Kędzierska-Matysek et al., 2013; Juszczak et al., 2018)**. With the rising addition of bee pollen, the Zn content in tested samples also significantly (p <0.05) increased, and for the addition of 25% it reached 2.73 mg.100g^{-1} (Table 2). The zinc content at the maximum bee bread concentration was 3.75 mg.100g^{-1} (Table 2). The similar zinc content in commercial samples of honey enriched with pollen was reported by **Grembecka and Szefer (2013)** and **Juszczak et al. (2018)**. According to **Grembecka and Szefer (2013)**, bee pollen is a rich source of zinc, and its content in this substance amounts to 2.9 mg.100g^{-1}. For the bee bread used as a honey ingredient, the increase of the zinc content was higher than in the case of pollen, and with the maximum supplementation, the content of that microelement reached 3.75 mg.100g^{-1}. As the two-way analysis of variance demonstrated, both the type and the level of the additive have a significant (p <0.05) influence on zinc content in enriched honey. Furthermore, a strong positive linear correlation (r = 0.9690) was found between ash and zinc content. An increase in zinc content following enrichment of honey with bee products significantly influences its nutritional value. While 100 g of multifloral honey covers ca. 2% of the daily zinc demand, enriched honey cover that demand in ca. 27% for pollen and ca. 37% for bee bread.

The copper content in both multifloral honey and honey enriched with bee products was the lowest of all mineral ingredients. In the multifloral honey, the copper content amounted to 0.10 mg.100g^{-1} (Table 2), and this corresponds to just 0.03% of all elements determined. The obtained results are similar to values reported by **Kędzierska-Matysek et al. (2013)** for Polish honey, and by **Kačániová et al. (2009)** for Slovak honey. As the conducted two-way analysis of variance has shown, both the type and the level of the additive have a significant (p <0.05) influence on the copper content in enriched honey (Table 2) and the addition of pollen or bee bread increases the content of the discussed element about five times. Furthermore, a strong positive linear correlation (r = 0.9603) was found, indicating a relationship between ash and copper content. The average reference human demand for copper is low and amounts to 1 mg. While 100 g of multifloral honey covers ca. 10% of the daily copper demand, enriched honey cover that demand in ca. 57% for pollen and ca. 46% for bee bread.

In the analyzed honey, the manganese content was at the level of 1.2 mg.100g^{-1} (Table 2), and this corresponds to 0.40% of the total mineral ingredients determined **(Grembecka and Szefer, 2013; Kędzierska-Matysek et al., 2013; Juszczak et al., 2018)**. The determined value is within the extensive range reported for Polish multifloral honey. An addition of bee pollen or bee bread to honey caused a significant (p <0.05) increase in the manganese content. For bee pollen, the manganese content increased about two times, while for bee bread it increased four times. The conducted two-way analysis of variance has shown that the manganese content in enriched honey significantly (p <0.05) depends both on the type and the level of the bee product added. Honey can be considered a very good source of manganese, as at the determined level of this ingredient, 100 g of honey covers 60% of the daily demand. Honey enrichment with bee products, which increased the manganese content, also caused honey's ability to cover the daily demand for that element to a level of 117% for pollen and as much as 246% for bee bread.

CONCLUSION

Based on obtained results it was found that the addition of pollen or bee bread to honey significantly influences the content of ash and selected macro- and microelements, excluding sodium. Significant positive linear correlations between the ash content and the content of some elements were observed. The greatest increase in mineral content was observed for magnesium, iron, and zinc. Enrichment of honey with the highest dose of bee pollen or bee bread resulted in an over 20-fold increase in the Mg and Fe content, and an over 14-fold increase in the Zn content. Honey enriched with the maximum addition of bee pollen was characterized by a higher content of K, Ca, Mg, Fe, and Cu compared to honey with bee bread. In turn, honey enriched with bee bread was characterized by a higher content of Zn and Mn. Due to a fact that both bee pollen and bee bread are good sources of minerals, their addition to honey significantly increases its ability to cover daily demand for macro- and microelements.

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SIMULTANEOUS DETERMINATION OF SWEETENERS AND PRESERVATIVES IN BEVERAGES BY HPLC-DAD-ELSD

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ABSTRACT

People suffering from diabetes or being overweight must severely reduce their sugar use, often seeking food with sweeteners. Often, sugar is replaced by non-nutritious sweeteners in beverages, which also contain several other substances like vitamins, caffeine, amino acids, phenolic compounds and thus increasing the shelf life of the beverages is additionally treated with the addition of preservatives. As the concentration of additives in food (including beverages) is determined by the legislation in force, it is necessary to have an appropriate analytical method for food control. Since artificial sweeteners and preservatives are very different substances, they are determined separately using different HPLC methods. In this work HPLC method combining the advantages of specific (diode array detector, DAD) and universal (evaporative light scattering detector, ELSD) detector was validated and used for simultaneous determination of benzoic acid, sorbic acid, aspartame, acesulfame K, saccharin, sucralose and steviol glycosides in sugar-free beverages. The proposed analytical method showed good linearity, precision, and accuracy. Measured limits of detection (0.6 – 11.8 mg.dm^{-3} depending on the analyte) were sufficient to analyze 5-times diluted beverage samples. The validated method has been successfully used for the simultaneous analysis of artificial sweeteners and preservatives in beverage samples (energy drinks, ice teas, carbonated drinks). Except for steviol glycosides, the concentration of monitored substances in beverages did not exceed the maximum permitted concentrations given in the valid legislation.

Keywords: sweeteners; preservatives; beverage; chromatography; DAD ELSD detectors

INTRODUCTION

Carbohydrates are the most important and quickest source of energy, accounting for more than half the energy value of our food. In addition to the natural carbohydrate content, foods are further sweetened with sugars and various sweeteners that give the products a pleasant sweet taste. Sweeteners are divided into intensive (non-nutritious) and bulk (nutritional) sweeteners **(Basoli and Merlini, 2003)**. Intensive sweeteners include both synthetic and natural sweeteners. The most commonly used are saccharin, sucralose, acesulfame K, stevioside, and rebaudioside A. The most popular bulk sweeteners are erythritol, sorbitol, xylitol, maltitol, isomalt, lactitol, and mannitol **(Mortensen, 2006)**. An increase in the number of autoimmune diseases, an ageing population, and above all, an unhealthy lifestyle is increasing the proportion of people suffering from diabetes. People with diabetes cannot use their blood glucose. This leads to a rise in blood sugar (hyperglycaemia) and other serious consequences **(Bartnik, Norhammar and Rydén, 2007)**. In addition to diabetes, excessive intake of refined sugars also poses a problem in terms of obesity and tooth decay **(Kamal, O´Toole and Bernabé, 2019)**. The use of sugar substitutes and intense sweeteners makes it possible to produce sweet foods for people suffering from diabetes while reducing the caloric value of the food at the same time it reduces the risk of obesity. The use of sweeteners in food products is governed by applicable national legislation. The list of permitted sweeteners in the Czech Republic is given in the Decree No. 122/2011.

Preservatives ensure the quality and safety of the product and prevent the adverse reactions that are responsible for food spoilage. At the same time, they inhibit the growth of undesirable microorganisms (bacteria, fungi, yeasts) and thereby prolong the shelf life of food during distribution and storage. Preservatives can be divided into natural, synthetic, and antibiotic **(Silva and Lidon, 2016)**. Preservatives used in the food industry must meet certain criteria. Preservatives and their metabolites must not be toxic or harmful. They should be readily soluble in water and have sufficient stability, even at higher temperatures. It must have antimicrobial properties within the pH range of a particular foodstuff. Preservatives should not affect the sensory properties of products and react with other food ingredients. The most common synthetic preservatives used in the food industry are benzoic acid, sorbic acid, or salts thereof.

Since the concentration of sweeteners and preservatives used in food production is limited by the laws in force it is necessary to monitor these substances in food and to have

the necessary analytical methods for this purpose. The most commonly used method for analysis of sweeteners and preservatives is high-performance liquid chromatography with UV or DAD detector **(Sik, 2012; Ha et al., 2013; Javanmardi et al., 2015; de Queiroz Pane et al., 2015)**. Since some substances absorb a small amount of radiation in the 200 – 700 nm range, universal detectors such as MS **(Yang and Chen, 2009; Di Donna et al., 2017)** or ELSD **(Wasik, McCourt and Buchgraber, 2007)** are also used. Due to the different nature of the substances, sweeteners and preservatives are usually determined using different HPLC methods separately. In this work HPLC method combining the advantages of specific and universal detectors was validated and used for simultaneous determination of benzoic acid, sorbic acid, aspartame, acesulfame K, saccharin, sucralose, and steviol glycosides in beverages.

Scientific hypothesis

By combining two detectors (DAD and ELSD) and by using HPLC it is possible to determine selected sweeteners together with preservatives in beverages using one method and one injection.

The concentration of sweeteners and preservatives used in sugar-free drinks complies with the limits set out in the applicable legislation.

MATERIAL AND METHODOLOGY

The individual standards of sweeteners and preservatives as well as formic acid, ammonium acetate, and triethylamine were purchased from Sigma-Aldrich (Germany). The purity of all standards and chemicals except stevioside and rebaudioside A was at least 99 %. The purity of stevioside was >95 % and purity of rebaudioside A was >96 %. Methanol, acetonitrile, and acetone (HPLC grade) were purchased from VWR (France). Ultrapure water with resistivity > 18 MΩcm was obtained from ELGA Purelab Classic UV (Veolia, France).

An Agilent 1260 liquid chromatograph with Poroshell 120 EC-C18 (4.6 x 150 mm, 2.7 μm) column, equipped with 1260 Infinity diode array detector (DAD) and 1260 Infinity evaporative light scattering detector (ELSD) was used in this study. The injection volume was 5 μL. The flow of the mobile phase was 0.5 $mL.min^{-1}$. The temperature in the column thermostat was 30 °C. The signal from the DAD detector was monitored at 210 nm. For ELSD detector nitrogen flow of 2 $dm^{-3}.min^{-1}$, nebulization temperature of 90 °C and evaporating temperature of 95 °C has been set.

Samples of sugar-free beverages were purchased from the supermarket located in Brno, Czech Republic. Four samples of energy drinks (S1 – S4), 2 samples of carbonated drinks (S5 – S6), and 2 samples of iced teas (S7 – S8) were purchased.

Statistical analysis

For each sample data analysis and statistical evaluation were performed in Microsoft Excel (Microsoft, USA) and XL-stat (Addinsoft, France, version 2014.5.03). Before the main data analysis, results were tested for outliners using the Grubbs test at significance level α=0.05).

RESULTS AND DISCUSSION

First, a suitable column was selected for the HPLC method to be tested. Non-polar C18 columns are most commonly used in the literature for the type of analysis required **(Grembecka et al., 2014; de Queiroz Pane et al., 2015; Sik, 2012)**. Thus, the end-capped Poroshell 120 EC-C18 column, which is packed with solid-core surface-porous microparticles and a porous silica gel outer layer, to which a non-polar dimethyl-n-octadecyl silane monolayer is bound, was chosen for the method being investigated.

The next step was to select the appropriate mobile phase. In the literature, mobile phases containing phosphate buffers are often mentioned for the analysis of sweeteners and preservatives **(Dossi et al., 2006; de Queiroz Pane et al., 2015; Zygler, Wasik and Namieśnik, 2009)**, however, the phosphate buffer is not compatible with the ELSD detector, and therefore a mobile phase with a different composition had to be chosen. Thus, the use of a mobile phase containing methanol, acetonitrile and 0.01 $mol.dm^{-3}$ ammonium acetate (mobile phase 1) and a mobile phase containing methanol (A), acetone (B) and a mixture of 0.02 $mol.dm^{-3}$ formic acid and 0.02 $mol.dm^{-3}$ triethylamine (C) (mobile phase 2) was investigated. Using Mobile Phase 1, separation of all analytes was not possible even by gradient adjustment. By using mobile phase 2, on the contrary, by optimizing the gradient, optimal separation of all analytes (except stevioside and rebaudioside A) was achieved. Stevioside and rebaudioside were mixed to one standard and quantified together as steviol glycosides (Figure 1 and Figure 2). The final gradient setting was: time 0 – 4 min 85% v/v C, 10% v/v A, 5% v/v B; time 4 – 10min 70% v/v C, 25% v/v A, 5% v/v B; time 10 – 15 min 60% v/v C, 35% v/v A, 5% v/v B; time 15 – 30 min 35% v/v C, 60% v/v A, 5% v/v B; time 30 – 40 min 85% v/v C, 10% v/v A, 5% v/v B.

In the following step, the linearity was verified. Calibration plots were constructed using mixed standards of 10, 25, 50, 100, 250, and 500 $mg.dm^{-3}$ (the 10 $mg.dm^{-3}$ standards were omitted for sucralose and steviol glycosides). Because the response function of the ELSD detector is known to be nonlinear, a logarithmic conversion for both concentration and peak area was performed. For all constructed calibration curves coefficients r^2 were >0.99 showing very good linearity in the concentration range tested.

The precision of the investigated method was verified by repeatability test during which a mixed standard of 10 $mg.dm^{-3}$ of the analytes of interest was repeatedly injected onto the column (steviol glycosides and sucralose concentration was 25 $mg.dm^{-3}$). Results from this test are presented in Table 1. The RSD values of the retention time were found to be <1%, the RSD of area and height of each analyte peak was found to be <2%.

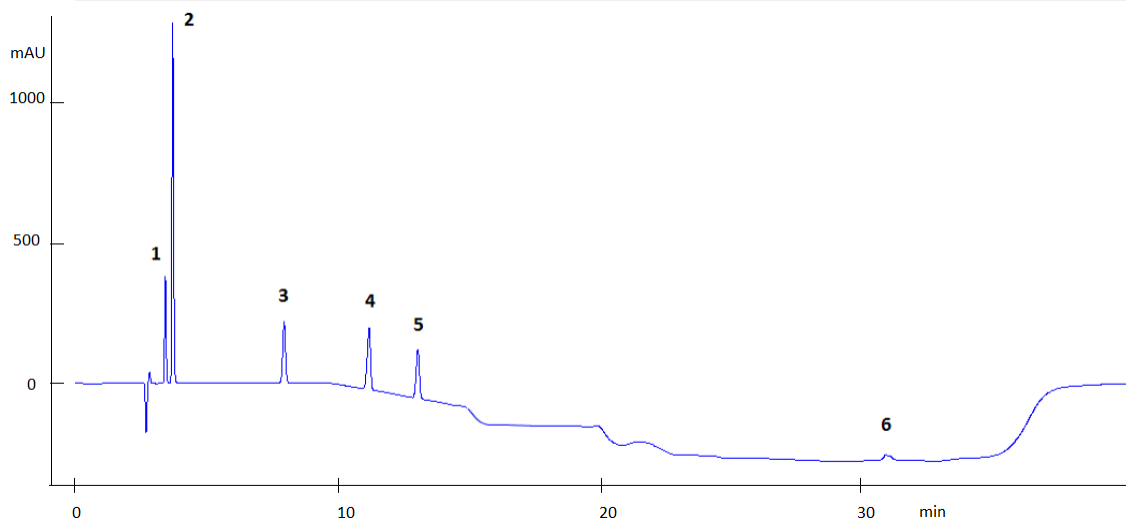

Figure 1 Chromatogram of standard (50 mg.dm^{-3}), DAD 210 nm.
Note: 1 = acesulfam K, 2 = saccharin, 3 = aspartam, 4 = benzoic acid, 5 = sorbic acid, 6 = steviol glycosides.

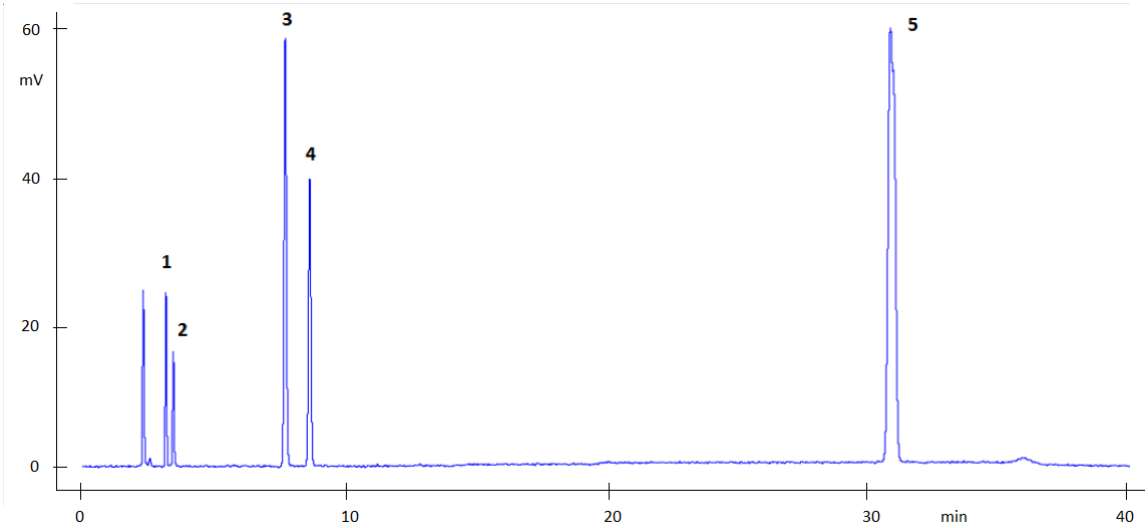

Figure 2 Chromatogram of standard (50 mg.dm^{-3}), ELSD.
Note: 1 = acesulfam K, 2 = saccharin, 3 = aspartame, 4 = sucralose, 5 = steviol glycosides.

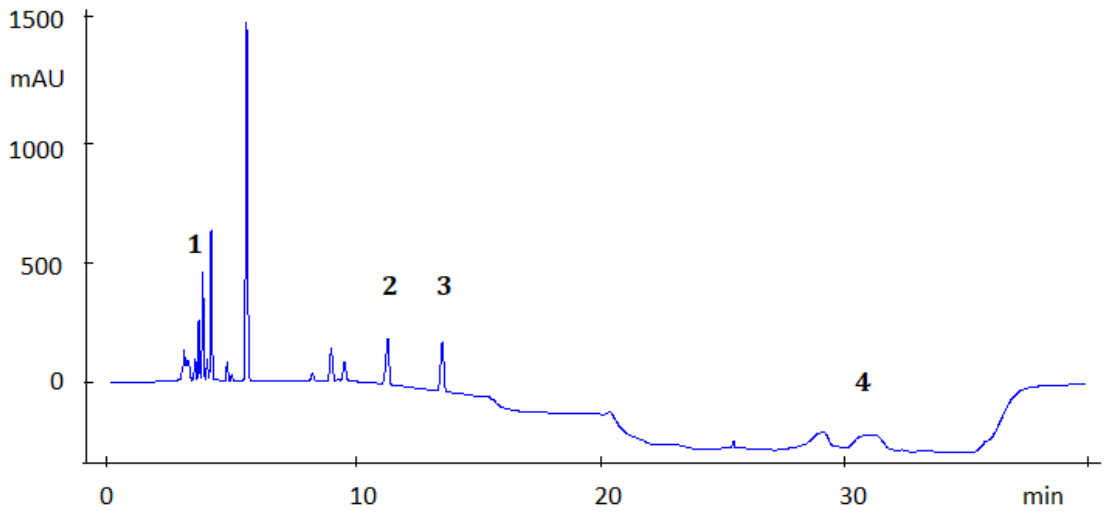

Figure 3 Chromatogram from the analysis of the real sample (S6), DAD 210 nm.
Note: 1 = acesulfame K, 2 = benzoic acid, 3 = sorbic acid, 4 = steviol glycosides.

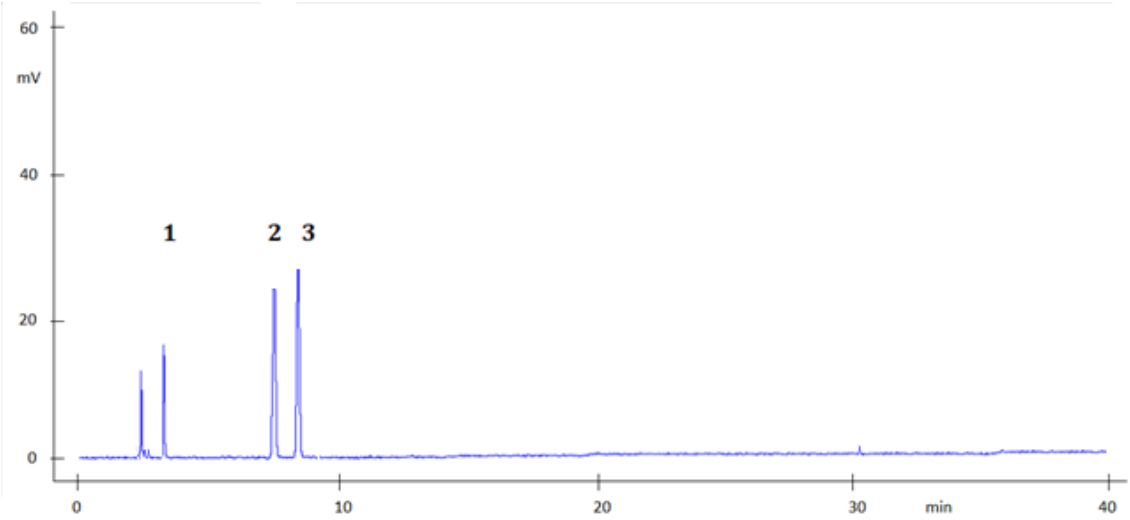

Figure 4 Chromatogram from the analysis of the real sample (S7), ELSD.
Note: 1 = acesulfame K, 2 = aspartame, 3 = sucralose.

Table 1 Repeatability of retention time (min), peak hight and peak area, (n = 6).

	Mean[a]	RSD[a]	Mean[b]	RSD[b]	Mean[c]	RSD[c]
ACS	3.46	0.37	119	0.75	457	0.86
SAC	3.70	0.47	352	0.36	1384	0.33
ASP	7.37	0.55	50	1.50	336	0.81
SUC	8.69	0.68	1.27	1.77	7.9	1.72
SG	31.20	0.17	9.8	1.70	57	1.72
BAC	11.73	0.53	57	1.42	471	0.93
SAC	12.82	0.53	52	1.06	431	1.26

Note: ACS = acesulfame K, SAC = saccharin, ASP = aspartame, SUC = sucralose, SG = steviol glycosides, BAC = benzoic acid, SAC = sorbic acid, RSD = relative standard deviation (%), [a] = Repeatability of retention time, [b] = Repeatability of peak hight, [c] = Repeatability of peak area.

Table 2 Concentration of sweeteners in analysed beverages.

Sample	sweeteners				
	ACS ($mg.dm^{-3}$ ±SD)	SAC ($mg.dm^{-3}$ ±SD)	ASP ($mg.dm^{-3}$ ±SD)	SUC ($mg.dm^{-3}$ ±SD)	SG ($mg.dm^{-3}$± SD)
S1	248 ±20	<0.6	122 ±7	<10	<12
S2	190 ±11	<0.6	120 ±4	<10	<12
S3	211 ±9	<0.6	<1.6	270 ±15	<12
S4	207 ±8	<0.6	159 ±7	<10	<12
S5	<0.9	<0.6	<1.6	<10	231 ±18
S6	278 ±12	<0.6	<1.6	75 ±6	222 ±14
S7	186 ±9	<0.6	120 ±10	173 ±13	<12
S8	191 ±10	94 ±5	103 ±8	<10	<12

Note: ACS = acesulfame K, SAC = saccharin, ASP = aspartame, SUC = sucralose, SG = steviol glycosides, S1 – S4 energy drinks, S5 – S6 carbonated drinks, S7 – S8 iced teas.

Table 3 Concentration of preservatives in analysed beverages.

Sample	preservatives	
	Benzoic ac. ($mg.dm^{-3}$ ±SD)	Sorbic ac. ($mg.dm^{-3}$ ±SD)
S1	<5.5	<4.4
S2	117 ±5	214 ±11
S3	120 ±9	223 ±15
S4	<5.5	<4.4
S5	159 ±8	180 ±7
S6	147 ±12	135 ±5
S7	<5.5	<4.4
S8	<5.5	104 ±9

Note: S1 – S4 energy drinks, S5 – S6 carbonated drinks, S7 – S8 iced teas.

Limits of detection and quantification were determined from calibration lines after repeated injection of a mixed standard of 10 mg.dm^{-3} of the analytes of interest (steviol glycosides and sucralose concentration was 25 mg.dm^{-3}) according to the method described by **Shirivastava and Gupta (2011)**. The limit of detection was determined to be 0.9 mg.dm^{-3} for acesulfame K, 0.6 mg.dm^{-3} for saccharin, 1.6 mg.dm^{-3} for aspartame, 10.4 mg.dm^{-3} for sucralose, 5.5 mg.dm^{-3} for benzoic acid, 4.4 mg.dm^{-3} for sorbic acid and 11.8 mg.dm^{-3} for steviol glycosides. Limit of quantification was determined to be 2.7 mg.dm^{-3} for acesulfame K, 1.9 mg.dm^{-3} for saccharin, 4.9 mg.dm^{-3} for aspartame, 31.4 mg.dm^{-3} for sucralose, 16.5 mg.dm^{-3} for benzoic acid, 13.3 mg.dm^{-3} for sorbic acid and 35.4 mg.dm^{-3} for steviol glycosides.

The accuracy of an analytical method was determined by performing a recovery test. The background concentration of analytes of interest in the sample used for the recovery test was 278 mg.dm^{-3} (acesulfame K), 75 mg.dm^{-3} (sucralose),
222 mg.dm^{-3} (steviol glycosides), 147 mg.dm^{-3} (benzoic acid) and 125 mg.dm^{-3} (sorbic acid). The sample was further spiked with all analytes at a concentration of 50 mg.dm^{-3} and then analysed again. The concentration of analytes in the sample after spiking was 319 mg.dm^{-3} (acesulfame K), 45 mg.dm^{-3} (saccharin), 48 mg.dm^{-3} (aspartame), 115 mg.dm^{-3} (sucralose), 278 mg.dm^{-3} (steviol glycosides), 245 mg.dm^{-3} (benzoic acid) and 171 mg.dm^{-3} (sorbic acid) which corresponds to recovery between 90 and 98%. Based on the measured results, it can be stated that the proposed method has very good accuracy.

After validation of the HPLC-DAD-ELSD method, this method was applied to the analysis of real samples. Results from the analysis are presented in Table 2 and Table 3 and the chromatogram obtained from the analysis of a real sample is shown in Figure 3 and Figure 4. Saccharin was detected only in one sample at the concentration of 94 ±5 mg.dm^{-3}. The most common sweetener in the beverages analyzed was acesulfame K, whose concentration ranged from 186 to 278 mg.dm^{-3}. The use of other sweeteners varied depending on the type of sample analyzed and their concentration in beverages was around 200 mg.dm^{-3}. Measured results are consistent with data published by other authors. **Sik (2012)** analyzed 56 soft drinks and only in 10 samples he detected the use of saccharin (27 – 78 mg.dm^{-3}). The concentration of acesulfame K in soft beverages is given in the literature in the range of 3 – 258 mg.dm^{-3}, the concentration of aspartame in the range of 27 – 559 mg.dm^{-3}, sucralose in the range of 13 – 152 mg.dm^{-3} and steviol glycosides in the range of 3 – 83 mg.dm^{-3}. **(Sik, 2012; Ha et al., 2013; Grembecka et al., 2014; de Queiroz Pane et al., 2015; Yongsun Lee et al. 2017; Di Donna et al., 2017)**. Not all samples contained preservatives. The measured concentration of benzoic acid in beverages was about 150 mg.dm^{-3}. Sorbic acid was found at a higher concentration. The concentration ranged from 104 to 223 mg.dm^{-3}. The measured concentrations are comparable with those published by other authors **(Grembecka et al., 2014)**, however, in some cases extremely high concentrations of monitored preservatives in beverages can be found **(Javanmardi et al., 2015)**. The sweeteners and preservatives identified in all samples examined corresponded to the composition on the product packaging. Except for steviol glycosides, the concentration of monitored substances in beverages did not exceed the maximum permitted concentrations given in the valid legislation. The maximum permitted concentration of steviol glycosides in beverages is 80 mg.dm^{-3}. This limit was exceeded by about three times in two samples.

CONCLUSION

The scientific hypothesis that by the combination of two detectors (DAD and ELSD) with HPLC it will be possible to determine sweeteners and preservatives in beverages using one method and one sample injection was confirmed. Using non-polar C18 stationary phase, mobile phase containing methanol, acetone, and a mixture of 0.02 mol.dm^{-3} formic acid and 0.02 mol.dm^{-3} trimethylamine were found as the most suitable. The proposed analytical method showed good linearity, precision, and accuracy. Measured limits of detection were sufficient to analyze 5-times diluted beverage samples. The concentration of monitored additives in beverages was following valid legislation. Only the amount of steviol glycosides in two samples was exceeded by about three times the maximum allowed content in beverages.

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MONITORING THE STABILITY OF FORTIFIED COLD-PRESSED SUNFLOWER OIL UNDER DIFFERENT STORAGE CONDITIONS

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ABSTRACT

The aim of the study was to evaluate the stability of cold-pressed sunflower oil mixed with different seeds and herb. The seeds and herb were added at 1% and 5% concentrations; samples were divided into 2 groups: stored in the dark and light. The primary products of oxidation and chlorophyll content were monitored during 3 months of storage. The results showed very low oxidation stability of experimentally produced cold-pressed oil mixtures/dressings, especially during storage on the light. The samples with 5% of hemp herb addition showed the best stability since they have peroxide value under 20 mekv $O_2.kg^{-1}$, both in the dark and on the light. Other samples, both stored in the dark and on light, were declared as not for human consumption due to high oxidative product development. The research represents an important storability evaluation of products that can be found on the market and that can be found very attractive for consumers.

Keywords: sesame; chia; hemp; oil dressings; storability

INTRODUCTION

The production of cold-pressed edible plant oils includes only pressing and occasional filtering of plant material rich in oil. Many consumers found this production attractive and this food commodity is becoming more popular. Especially because in this kind of edible oil production, organic solvents are prohibited. The advantage of cold-pressed oil is that natural beneficial components, that are present in the raw material, are preserved **(Febrianto and Yang, 2011; Parker et al., 2003)**.

Sunflower has good tolerance to drought and different soil typology. These properties have made sunflowers to be one of the most important oilseeds in the world, both in developed and developing countries. The production of cold-pressed oil has been increasing due to the chance for smaller farmers to produce it, while the demand for it is increasing too **(Foppa Pedretti et al., 2019)**. On the other hand, edible plant cold-pressed oil has low oxidative stability. The fortification of this food commodity should lead to longer shelf life and consequently the better oxidative stability **(Mazaheri et al., 2019)**.

Cold-pressed oils, including sunflower cold-pressed oil, are a good source of polyphenols, chlorophylls, and carotenoids. These compounds have beneficial properties for humans since they have anti-inflammatory, anticancer properties, same as antioxidant and antibacterial activities **(Song et al., 2019; Wu et al., 2019)**. The total area in the Czech Republic used for the planting of oilseeds is 412 060 ha **(Zehnalek, 2019)**.

Food fortification has been gaining in popularity recently. Chia seeds are often called "superfoods" due to the high nutritional value for humans. Chia, hemp, and sesame seeds can be used for the fortification of cold-pressed plant oils since they have a beneficial nutritional composition **(Urbizo-Reyes et al., 2019; Bartkiene et al., 2019; Kermani et al., 2019)**.

On the other side, the quality and stability of these seeds fortified cold-pressed oils are questionable. The study aimed to monitor oxidative-hydrolytic stability and nutritional profile of cold-pressed sunflower oil fortified with o chia, sesame, and hemp seeds, as well as hemp herb.

Scientific hypothesis

The fortification influence on the stability of cold-pressed sunflower oil. Since chlorophylls can act as antioxidants and prooxidants independence on storage conditions we are expecting different stability properties of experimentally produced oil mixtures/dressings.

MATERIAL AND METHODOLOGY

The Velox cultivar of sunflower (*Helianthus annuus*) was cultivated in the Czech Republic and was obtained from the company Olejářství z Horňácka, Uherské Hradiště. Sunflower seeds were harvested and stored in a dry cellar until pressing. After milling the oil was stored in the dark until mixing with seeds and herb (15 days).

Table 1 The samples description.

Samples	Sample description
1% SS1	1% sesame seeds
5% SS5	5% sesame seeds
1% SKS1	1% hemp seeds
5% SKS5	5% hemp seeds
1% SCH1	1% chia seeds
5% SCH5	5% chia seeds
1% SKC1	1% hemp tea
5% SKC5	5% hemp tea
SK	without addition of seeds/tea

Table 2 The measured parameters in sunflower samples at the beginning of the experiment.

	Peroxide value (mekv $O_2.kg^{-1}$)	Free fatty acids mg $KOH.g^{-1}$	Total phenol content $mg.mL^{-1}$	Chlorophyll content ppm
Sunflower oil	8.20 ±0.87	0.07 ±0.01	0.23 ±0.01	2.78 ±0.19

The sesame seeds (*Sesamum indicum* L.), hemp seeds (*Cannabis sativa* L.), and chia seeds (*Salvia hispanica* L.) were obtained from the retail shop "Zdraví z přírody" in Brno, the Czech Republic. The hemp herb was obtained from the retail shop "Sativa-Medical" in Brno, the Czech Republic.

The sesame/chia/hemp seed and hemp herb were added to the sunflower cold-pressed oil in the following concentrations: 1% and 5%. The samples were stored in glass bottles and divided into two groups, one group was covered with aluminum foil and stored in the dark; another group was exposed to the daylight, without aluminum covering. Samples were stored at 25 °C at the Department of Vegetable Foodstuffs Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology, the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (GPS: 49°12'59.2"N 16°35'41.0"E). The control samples were without seed/herb addition. The samples were analyzed at the beginning of the experiment and after 1 and 3 months of storage. The description of the samples is given in Table 1.

The peroxide value (PV) was determined according to **ISO 3960 (2017)** standard. 5 g of the sample was mixed with 30 ml of a mixture of chloroform and glacial acetic acid (2:3 ratio). The mixture was shaken for 1 minute, then 30 mL of distilled water and 5 mL of 1% starch solution were added. The sample was titrated with 0.01M $Na_2S_2O_3$. The blank sample was prepared in the same way with the addition of water instead of the sample.

The determination of chlorophylls was performed by the spectrophotometric method according to **Kraljić et al. (2013)**. The absorbance of chlorophylls in oil was measured at 670, 630, and 710 nm against cyclohexane (blank sample) by spectrophotometer (CE7210 DIET-QUEST, Cambridge, England).

Statistical analysis

Statistical significance at $p < 0.05$ was evaluated by one-way ANOVA analysis of variance, and parametric Tukey post hoc test (in the case when Levene's test showed equal variances $p > 0.05$) and nonparametric Games–Howel post hoc test (in the case when Levene's test showed unequal variances $p < 0.05$) for finding differences within groups. Overall differences among samples were checked by principal component analysis (PCA) using SPSS 20 statistical software (IBM Corporation, Armonk, USA).

RESULTS AND DISCUSSION

Table 2 is showing the results of the samples (cold-pressed sunflower oil samples without seeds and herb) at the beginning of the experiment.

The amount of primary products of oxidation, represented as peroxide values (mekv $O_2.kg^{-1}$), in the samples of sunflower oil (mixtures, same as without seeds/herb addition) after one month of storage can be seen from Table 3. The results are clearly indicating the high influence of storage conditions on the formation of hydroperoxides. After one month of storage, both on light and in the dark, only SKC5 (5% of hemp herb addition) samples showed better oxidation stability than control samples (SK).

3 months of storage showed an even higher oxidation ratio. Significant ($p < 0.05$) changes occurred in almost all oil mixture. The significant differences between samples stored in the dark and in light can be also seen from the principal component analysis (Figure 1). Out of all investigated samples only sample SKC5, stored in the dark, had a lower peroxide value than 20 mekv $O_2.kg^{-1}$. All other samples had peroxide value over 20 mekv $O_2.kg^{-1}$. This finding is making these samples be declared as not for human consumption.

The peroxide value, as the method for the determination of primary oxidation products, can be significantly influenced by the presence of antioxidants, such as phenols and chlorophylls. Chlorophylls have been proven to be good antioxidants in cold-pressed olive oil, but only during the storage in the dark. When cold pressed oil is stored on light, chlorophylls were found to act as prooxidants and they even make food matrix to oxidize more rapidly.

Table 3 Determination of peroxide value (mekv $O_2.kg^{-1}$).

Samples	Storage in dark		Storage on light	
	After 1 month	After 3 months	After 1 month	After 3 months
1% SS1*	12.06 ±1.32^{a**}	41.13 ±0.38^{b}	67.08 ±1.84^{c}	186.76 ±0.62^{d}
5% SS5	10.18 ±0.58^{a}	35.49 ±0.01^{b}	37.77 ±2.72^{ac}	123.78 ±0.32^{b}
1% SKS1	10.48 ±0.45^{a}	42.86 ±0.69^{b}	32.40 ±0.53^{c}	134.92 ±0.90^{d}
5% SKS5	12.56 ±0.87^{a}	60.29 ±0.09^{b}	52.96 ±0.26^{c}	169.29 ±1.23^{d}
1% SCH1	20.77 ±0.84^{a}	62.47 ±0.40^{b}	24.78 ±0.84^{a}	108.93 ±0.99^{c}
5% SCH5	21.08 ±0.46^{a}	62.54 ±0.55^{b}	26.60 ±0.72^{c}	125.88 ±0.30^{d}
1% SKC1	13.24 ±1.4^{a}	50.34 ±0.90^{b}	51.46 ±6.03^{ab}	209.24 ±0.11^{c}
5% SKC5	5.99 ±0.09^{a}	10.33 ±0.86^{a}	3.47 ±3.51^{a}	73.04 ±2.28^{b}
SK	8.96 ±0.29^{a}	62.98 ±0.15^{b}	17.98 ±1.42^{a}	80.42 ±0.29^{c}

Note: *Samples description is shown in Table 1; **lowercase letters shown statistically significant difference (p <0.05) between columns.

Table 4 Determination of chlorophyll content (ppm).

Mycotoxins	Storage in dark		Storage on light	
	After 1 month	After 3 months	After 1 month	After 3 months
1% SS1*	2.35 ±0.10^{a**}	2.19 ±0.04^{b}	0.28 ±0.00^{c}	0.24 ±0.00^{d}
5% SS5	0.13 ±0.09^{ac}	3.27 ±0.28^{c}	0.37 ±0.00^{b}	0.32 ±0.00^{da}
1% SKS1	2.35 ±0.04^{a}	1.49 ±0.02^{b}	0.27 ±0.00^{c}	0.22 ±0.00^{d}
5% SKS5	2.34 ±0.05^{a}	1.28 ±0.01^{c}	0.26 ±0.00^{d}	0.22 ±0.00^{c}
1% SCH1	3.15 ±0.04^{a}	0.70 ±0.03^{b}	0.27 ±0.00^{c}	0.26 ±0.00^{d}
5% SCH5	3.82 ±0.12^{a}	1.49 ±0.03^{d}	0.25 ±0.00^{c}	0.21 ±0.00^{c}
1% SKC1	2.50 ±0.06^{a}	1.72 ±0.09^{b}	0.26 ±0.00^{c}	0.21 ±0.00^{d}
5% SKC5	3.00 ±0.05^{a}	1.97 ±0.08^{a}	0.28 ±0.00^{b}	0.21 ±0.00^{c}
SK	2.47 ±0.12^{a}	1.57 ±0.05^{b}	0.28 ±0.00^{c}	0.24 ±0.00^{d}

Note: *Samples description is shown in Table 1; **lowercase letters shown statistically significant difference (p <0.05) between columns.

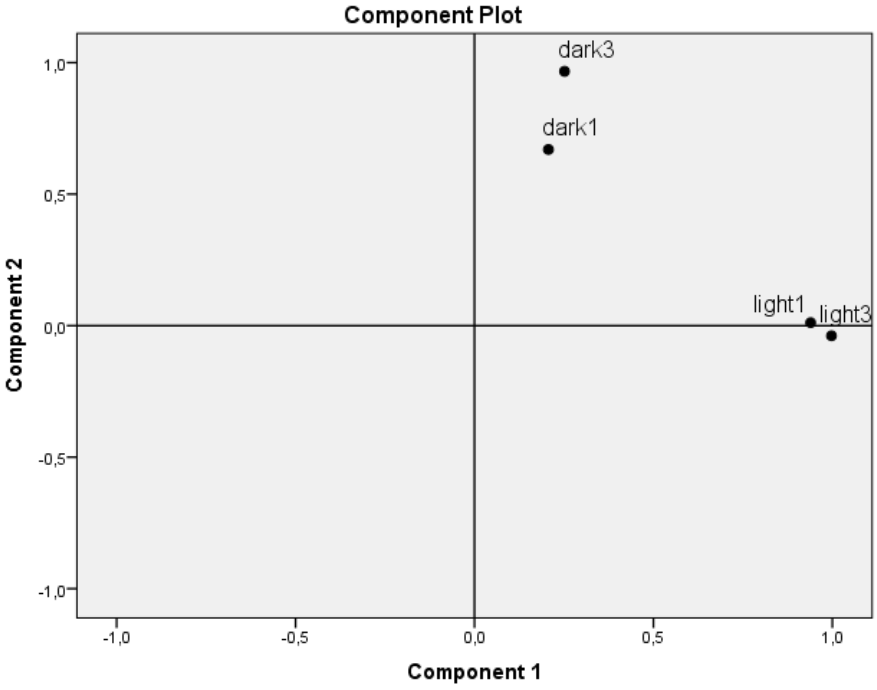

Figure 1 Principal component analysis (PCA) of samples‘ peroxide values. Note: dark1 – the samples stored 1 month in the dark; dark3 – the samples stored 3 months in the dark; light1 – the samples stored 1 month on light; light3 – the samples stored 3 months on light.

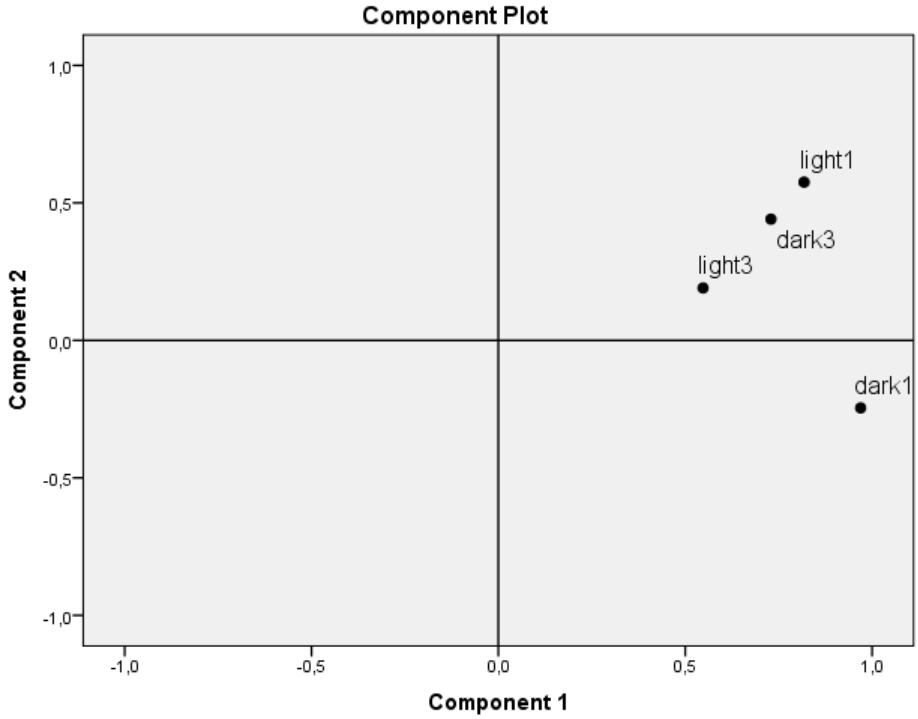

Figure 2 Principal component analysis (PCA) of chlorophyll contents in samples stored in the dark and light. Note: dark1 – the samples stored 1 month in the dark; dark3 – the samples stored 3 months in the dark; light1 – the samples stored 1 month on light; light3 – the samples stored 3 months on light.

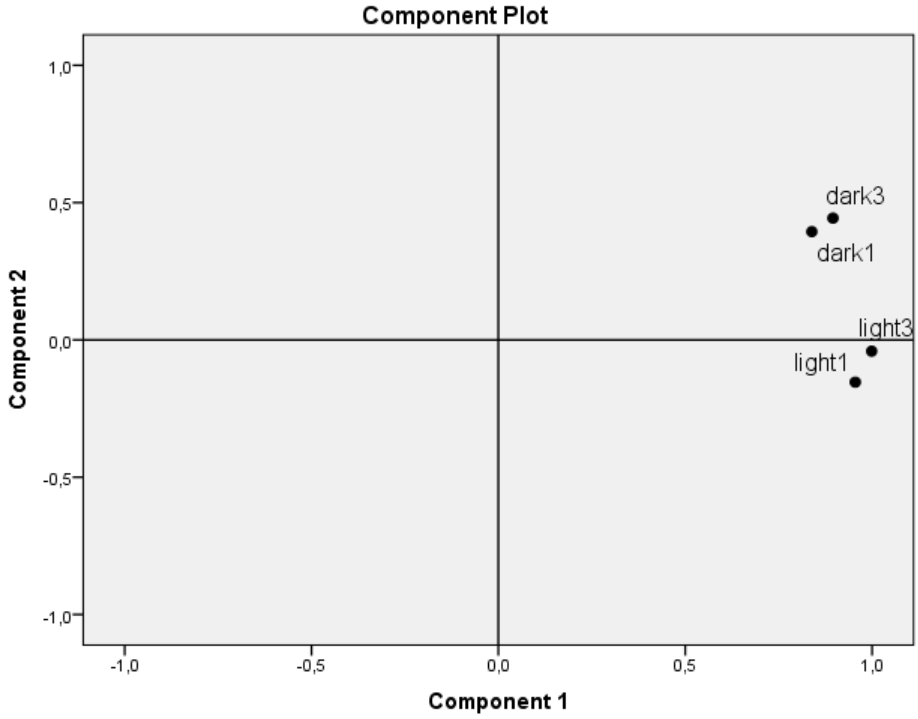

Figure 3 Principal component analysis (PCA) of overal differences between samples stored in the dark and on light dark1 – the samples stored 1 month in the dark; dark3 – the samples stored 3 months in the dark; light1 – the samples stored 1 month on light; light3 – the samples stored 3 months on light.

That is the main reason for the better oxidation stability of refined edible plant oils, since polyphenol, carotenoid and chlorophyll compounds are removed during the production **(Choe and Min, 2006)**. The results are emphasizing that cold-pressed sunflower oil has a very short shelf life since the majority of the investigated samples developed rancid taste. Rancidity is developed when peroxide value is over 20 mekv $O_2.kg^{-1}$ **(Ekwenye, 2006)**. Oxidation stability is the main property of edible plant oil and our results indicated very low stability of cold-pressed sunflower oil. The fortification/addition of different seeds and herb did not affect positively oxidation stability. Oppositely, the fortification resulted in higher oxidation degradation, especially after 3 months of storage on light (Table 3). Photooxidation, which influences the oxidation processes of samples stored on light, is producing singlet oxygen that is more reactive than triple oxygen (produced by auto-oxidation) and leads to faster oxidation. Sunflower can be declared worldwide as the most consumable edible oil. Though, the disadvantage of sunflower oil, especially cold-pressed, is its low oxidative stability **(Caponio et al., 2005)**.

Table 4 is showing the ratio of chlorophyll content in sunflower oil, both fortified and not fortified, during 3 months of storage on light and in the dark. It can be seen the amount of chlorophyll degradation during the storage period. The samples stored on light had significantly (p <0.05) lower chlorophyll content than samples stored in the dark. The degree of chlorophyll degradation is also clearly visible from the principal component analysis, where the sample stored one month in the dark formed one group and differ from the rest samples (Figure 2). The distinguish between samples stored in the dark and on the light during 3 months of storage are also visually observable from Figure 3. The results of the samples stored in the dark and in light, analyzed by principal component analysis, formed separate groups. A certain amount of chlorophylls occurs in cold-pressed vegetable oil, while these contents are removed in refined oils by a refining process **(Szydłowska-Czerniak et al., 2019)**. Chlorophyll contents decrease during longer storage periods and with higher storage temperatures **(Rasul and İnanc, 2014; Islam et al., 2019)**. Under light chlorophylls degrade more rapidly than in the dark **(Lee et al., 2014)**. Chlorophylls are affecting the beneficially nutritional profile of oils and also oil color. The auto-oxidation is triggered when pigments are exposed to light or heat. Chlorophylls are capable to transform electromagnetic radiation energy to triplet oxygen and produce highly oxidative singlet oxygen. Consequently, unsaturated fatty acids react with these free radicals. In this way, the quality of the oil is degraded **(Diaz et al., 2019)**. During auto-oxidation primary products of oxidation, hydroperoxides are triggering free radical formation reaction and off-flavor products. These off-flavor products are formed by the release of short-chain fatty acids during oxidation **(Kiritsakis and Markakis 1984; Kiritsakis, 1995)**.

Chlorophylls are strong antioxidants, but when oil is stored in the dark since on light they have acted as prooxidants **(Choe and Min, 2006)**. That is the reason for the following requirement: edible plant refined oil should have chlorophyll content under 1 $mg.kg^{-1}$ **(Szydłowska-Czerniak et al., 2019)**. The same trend of lowering chlorophyll content during 2 months of storage, under light, was noticed with cold-pressed olive oil **(Caponio et al., 2005)**.

CONCLUSION

The study emphasized cold-pressed edible plant oils storage issues, such as sunflower oil. The study unambiguously indicates that the storability/stability of cold-pressed sunflower oil is highly questionable. Fortification with seeds and herb did not affect significantly (p <0.05) experimentally produced oil mixtures/dressings since oxidation even in fortified samples were over the consumption limit. Certain, slightly better oxidation stability was noticed in fortified samples during 3 months storage in the dark. The only samples with peroxide value below 20 mekv $O_2.kg^{-1}$ (the palatable limit) were samples prepared with 5% of the hemp herb. The study showed very low oxidation stability of cold-pressed sunflower oil; the fortification was reasonable only in the samples with the addition of 5% hemp tea, only these samples stayed significantly more stable in comparison with other experimentally produced samples. The study emphasized the rapid degradation nature of chlorophylls during storage under the light. Since these kinds of products can be already found on the market and can be found attractive for consumers, our study represents valuable information for producers and consumers.

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RESEARCH OF SELECTED PHYSICAL INDICATORS OF TABLE EGGS IN THE SMALL-SCALE BREEDINGS FROM THE ASPECT OF HEALTH SAFETY

Mária Angelovičová, Michal Angelovič, Lucia Zeleňaková

ABSTRACT

The purpose of this study was to investigate selected indicators of the table eggs in small-scale breedings, focusing mainly on the eggshell and its contamination and damage. Our object of study was eggs, shell, damage, and contamination of table eggs. Four small-scale breedings were randomly selected in Slovakia. These breeds were alternatively with an outdoor free-range. Laying hens Dominant was bred under conditions small-scale breeds No.1, No. 2 and No. 3 in the 1st laying cycle, and No. 4 in the 2nd laying cycle. Egg weight was balanced in three small-scale breedings. Egg weight was significantly higher in the fourth small-scale breeding, statistically significant (p <0.05) compared to egg weight in the studied 3 small-scale breedings. Shell weight and shell thickness in the equatorial plane of the egg were balanced in three small-scale breedings and in the fourth small-scale breedings were significantly higher, statistically significant (p <0.05). The higher egg weight per breeding is related to the higher laying hens age that was in the 2nd laying cycle compared to laying hens 3 small-scale breedings in the 1st laying cycle. Higher eggshell weight in three farms may be related to improved conditions in breeding hygiene, as confirmed by the results of investigations into contamination and damage to table eggs. These differences may also be related to nutrition.

Keywords: small-scale breeding; egg; eggshell; contamination; damage

INTRODUCTION

Improved animal welfare is the sum of physical and mental well-being. Many factors affect the welfare of laying hens. The results obtained from research into improved living conditions may be contradictory. In this context, experts agree that a suitable approach to assessing the welfare of laying hens is to integrate the information across disciplines, using several different methodologies **(Scientific Panel on Animal Health and Welfare, 2005)**.

The assessment of the indicators of egg external quality raised laying hens in the system of alternative environments, such as on litter, is fundamental for the promotion of this rearing system. To determine the effects of the rearing environment on the performance and welfare of hen laying, the analysis of productive parameters and egg quality and safety are examples of some measures adopted **(Alves, Silva and Piedade, 2007)**.

According to the knowledge previously published, it is known that laying hens kept in domestic conditions (small-scale breeding) they largely preserve kinds of natural behavior, generally according to their wild ancestor **(Fraser and Broom, 1990)**.

Laying hens have been bred for several thousand years in some properties. Domestication and selection took place. Some types of behavior originate in genetics and persist in the environment, that it requires to prepare conditions for satisfying hen laying needs. This type of behavior is known as instinct. Ethologists (scientists who specialize in animal behavior studies) explain that, in terms of motivation and ethological needs, strongly motivated behavior is largely controlled by internal factors (such as changes in hormone levels), which are available regardless of the type of outdoor environment **(Duncan, 1998)**.

Behavior identified as important for improved welfare laying hens includes nesting, examination, perch, raking and nutritional behavior, dusting, engaging in comfortable behavior (such as over lighting, etc.) **(Petherick and Rushen, 1997)**.

Laying hens are biologically able to adapt to environmental conditions when the environment is appealing to them. At that time, they increase interest in

such an environment, which in turn increases the quality of their living conditions. The environment is engaging, increases interest, and adds to the quality of animal life.

A rich and diverse environment stimulates exploratory behavior and allows pecking and raking **(Knierim, 2006)**.

According to **Baer (1998)**, an enriched environment has a positive impact on the physical, mental, and social well-being of animals, including laying hens and can improve their health. European Food Safety Authority, Panel on Animal Health and Welfare (AHAW), an independent an advisory body providing the scientific basis for European policy and legislation, based on the processing of scientific literature, it has come to the following conclusion: stabling systems differ in the possibilities for laying hens to show species-specific behavior, such as raking, dusting, exploring and selecting a suitable nest. Sufficient space must be provided for laying hens, to carry out the above-mentioned natural activities. A free-range breeding system in nature can pose a risk of laying hens and endanger their health. Layers in outdoor free-range may be exposed to wild birds, insects, and other potentially infectious agents **(Scientific Panel on Animal Health and Welfare, 2005)**.

The laying hens may come into contact with bacteria and intestinal parasites and coccidia **(McDougald, 2003; Scientific Panel on Animal Health and Welfare, 2005)**.

The object of social interest in the context of the welfare of laying hens, it is largely focused on farm conditions, most for breeding systems, conditions for natural behavior, and limited conditions associated with stress and mutilation. However, the impact of genetics on the welfare of laying hens is clear, with strong genetic effects on traits including immune function **(Bridle et al., 2006)**, bone strength **(Stratmann et al., 2016; Candelotto et al., 2017)**, feather pecking, feather condition and associated mortality **(Su et al., 2005; Brinker et al., 2014; Muir et al., 2014)** and fear **(Uitdehaag et al., 2008; de Haas et al., 2014)**.

Bacteria belong to the main cause of human foodborne diseases v worldwide and infected poultry flocks are the most common cause of human infection through the storage of foods.

Human salmonellosis is more often associated with the consumption of poultry and poultry products, including eggs, than with the consumption of food from other animals. All producers of table eggs, regardless of the type of breeding system, are subject to strict safety requirements **(Gast, 2003)**.

De Reu et al. (2006) note that the high risk of transmission of infection to table eggs is the higher the microbial contamination in the environment, such as in *Salmonella enteritidis*.

De Knegt et al. (2015) reported that in a laying hen flock, it was caused by human Salmonella as the main source of infections. They attributed approximately 40% of all Salmonella cases to *Salmonella enterica* serovar Enteritidis.

The incidence of human *S. enteritidis* infections is related to the prevalence of this pathogen in commercial flock eggs **(Arnold et al., 2014)**.

For extensive implementation, comprehensive risk reduction programs and testing of laying hens in flocks intended for the production of table eggs are attributed to a reduction in the incidence of human *S. enteritidis* infections **(Wright et al., 2016)**.

Verhallen-Verhoef and Rijs (2003) reported that hygiene in breeding conditions is one of the most important factors for laying hens. If there is a large number of laying hens in a small area, it is a great problem to maintain hygiene and then the hens are exposed to a lot of stress.

Otter (2015) notes that in the conditions of the small-scale breedings there is common breeding with a free-range system, which has proved its worth. In the breeding area, it is very appropriate to provide the facilities necessary for carrying out the natural activities of the laying hens, e.g. such as perch for rest, litter material for raking, and others. Hygiene and cleanliness in the breeding environment are the basis for the good health of laying hens, but also for the laying of non-harmful eggs concerning the consumer. The application of welfare aspects is also important for laying hens under small-scale conditions. These aspects support the healthy development of laying hens and the production of quality and health-safe table eggs.

In the **Council of the European Union (2006)** it was noted that table eggs are sold worldwide. On the European Union market, eggs are classified in quality class A or quality class B. Quality class A is classified for direct human consumption. On the contrary, class B eggs are marked as technical and are not intended for direct human consumption. Laying hen nutrition and post-laying egg handling are factors that play a very important role in determining the safety and quality of table eggs. The eggshell is characterized by being a natural external packing table of laying hens, the task of which is to prevent the penetration of contaminants into the internal egg content. The system of rearing, but also the type of feed administered by laying hens, affects egg composition to a very large extent.

Surai and Sparks (2001) report that there is a lack of knowledge about factors of the table egg chemical composition concerning a free-range or a range consisting of grassland.

Eggshell quality has a major economic impact on quality egg production because broken and cracked eggs mean an economic loss for farmers **(Yoho et al., 2008)**.

The abnormalities can be observed sometimes on the egg surface, on the shell. Eggshell surface abnormalities are assessed by altered shell surface, shell dilution, increased translucence, cracks, and cracks in the eggshell. These abnormalities, changes in quality and ultrastructure have been observed in flocks of hen laying in the experiment by **(Kursa et al., 2019)**.

The purpose of this study was to investigate selected indicators of the table eggs in small-scale breedings, focusing mainly on the eggshell and its contamination and damage.

Scientific hypothesis

Scientific hypothesis: balanced results selected indicators of table eggs in small-scale breedings, due to the small numbers of animals in breeding and outdoor free-range for carrying out natural activities.

MATERIAL AND METHODOLOGY

Object of research

Our object of study was eggs, shell, damage, and contamination of table eggs. Four small-scale breedings were randomly selected in Slovakia. These breeds were alternatively with an outdoor free-range.

Rearing conditions of the laying hens

Laying hens Dominant was bred in conditions of 4 small-scale breeders in Slovakia. Breeding conditions as well as nutritional conditions were ensured in these small-scale breedings of small-scale breeds in accordance with laying hens needs. Laying hens Dominant was reared in small-scale breedings No. 1, No. 2, and No. 3 in the 1st laying cycle, and No. 4 in the 2nd laying cycle. Henhouse with deep litter and free-range was a breeding house for laying hens. Laying hens had the opportunity to run daily in the summer from 6:00 am to about 7:00 pm. pm and in winter until 5:00 pm. The hen house equipment consisted of a watering-place, a feeder, a nest, and perch. To lay eggs, a nest was made for them to be made by hand collection. Drinking water and feed were part of the free-range. Laying hens were fed with a conventional feed mixture intended for laying hens, which was replenished at least 2 times a day. Sometimes laying hens were fed with food from the kitchen or crushed eggshells. Drinkers and feeders were washed daily. The eggs produced were harvested once a day in the summer in the afternoon and twice a day in the winter in the morning and afternoon.

Egg samples of 80 pieces were obtained from four selected small-scale breeders, i.e. 20 eggs from each small-scale breeder. Investigation of egg samples was carried out in a laboratory at the Department of Food Hygiene and Safety.

Characteristics to be collected on egg samples

Physical indicators of table eggs from small-scale breeders No. 1, No. 2, No. 3 and No. 4:

- the weight of egg – KERN PLE scales, max. 420 g, d = 0.001 g,
- the weight of eggshell – KERN PLE scales, max. 420 g, d = 0.001 g, dried eggshells in a drier at a temperature of 55 °C,
- shell thickness in 3 parts of the equatorial plane of the egg – DIAL INDICATOR, max. thickness 30 mm, d = 0.01 mm, dried eggshells in drier at 55 °C.

Contamination and egg damage under the light of a 100W table lamp from small-scale breedings 1, No. 2, No. 3, and No. 4: blood spots, droppings, pigment dots, other deposits, calcium deposits, bumps on the surface, and deformed egg shape.

Figure 1 Laying hens in the free-range.

Figure 2 Breeding equipment of hen house.

Statistical analysis

The results in the study are presented as mean – arithmetic mean ($\bar{x}$), variance range (R), which determines the difference between the minimum value (Min) and the maximum value (Max), the standard deviation (*SD*), and the coefficient of variation (cv, %).

Hypotheses about equality of mean values were tested using a one-factor analysis of variance (F) at significance levels $\alpha = 0.05$, $\alpha = 0.01$ and $\alpha = 0.001$. One-factor variance analysis (ANOVA) is the simplest form of ANOVA that examines the relationship between interval and nominal variables. It tests the null hypothesis of the mean equivalence, assuming that the selections have the same variance. The null hypothesis indicates that there is no relation between the interval and the nominal variable. If the calculated statistical value F is greater than the corresponding character value that divides the statistical set of a group with the same number of Fisher-Snedecor distribution elements FI-1, n-I, the hypothesis of equality of mean values is rejected.

Scheffe's test was used at a significance level of $\alpha = 0.05$ to compare the difference in the indicator between small-scale breedings. The Pearson correlation coefficient (r) reflects the relation between the two egg variable variables. The Pearson correlation coefficient (r) reflects the degree of the linear relation between the data of the two egg indicators. Its value is between -1 and +1. A +1 indicates that there is a high positive linear relationship between the two indicator data. A value of -1 means that there is a high negative linear relation, and value of 0 means that there is no linear relation between the two indicator data. The interpretation of the size of the correlation coefficient is given by **Cohen (1988)**.

Values of correlation coefficient (r) and strength of dependence between two variables: below 0.1 trivial (simple, light), 0.1 – 0.3 weak, 0.3 – 0.5 medium, above 0.5 strong. It is often reported in the publications that the correlation coefficient values of 0.7 – 0.9 represent a very strong relation and 0.9 – 1 as an almost perfect relation between two variables. The correlation coefficient results are statistically significant at $\alpha = 0.05$, $\alpha = 0.01$ and $\alpha = 0.001$. The SAS statistical package, version 8.2, was used to statistically evaluate the results.

RESULTS AND DISCUSSION

Egg weight

Average egg weight in individual small-scale breedings is given in Figure 3. Statistically evaluation of egg weight in individual small-scale breedings is given in Table 1.

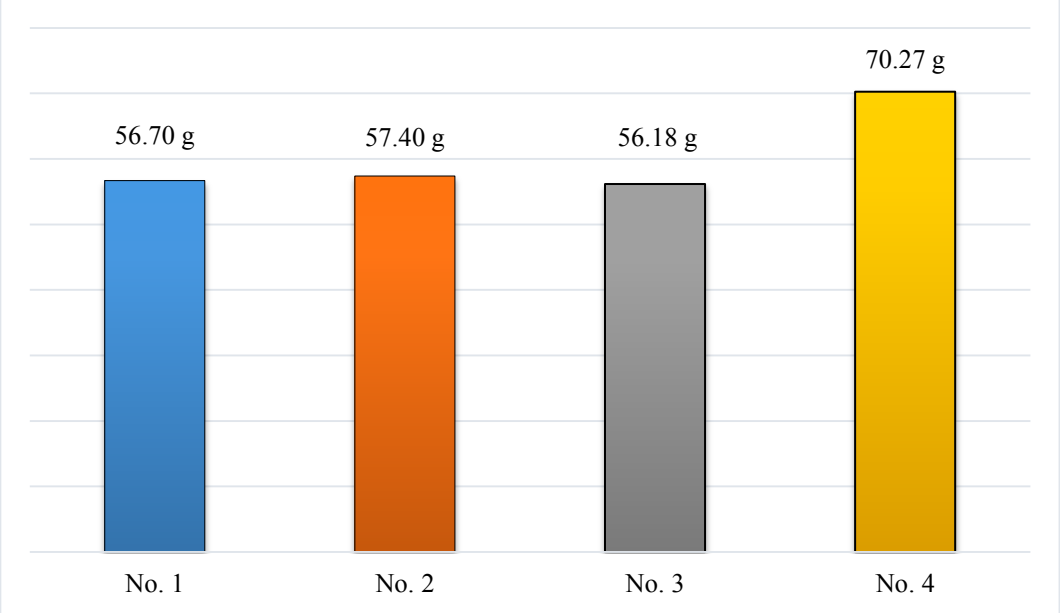

Figure 3 Average egg weight in individual small-scale breedings No. 1, 2, 3 and 4, g.

Table 1 Statistical evaluation of egg weight in individual small-scale breedings No. 1, 2, 3, and 4.

Small-scale breeding	F-test 61.97^{+++}				Scheffe´s test $p_{0.05}$		
	n	*SD*	c_v, %	R, g	No. 2	No. 3	No. 4
No. 1	20	3.03	5.35	53.08 – 64.11	-	-	+
No. 2	20	4.36	7.59	46.78 – 65.45		-	+
No. 3	20	4.07	7.25	51.98 – 66.72			+
No. 4	20	3.81	5.42	62.88 – 78.79			

Note: n – multiplicity; *SD* – standard deviation; c_v – coefficient of variation; R – variation range as the difference between the smallest and the largest value of the data distribution; +++: statistically significant difference among group means by analysis of variance (p <0.001); +: statistically significant difference among groups by Sheffe´s test (p <0.05); –: no statistically significant difference among groups by Sheffe´s test (p >0.05).

The average egg weight was found to be either the same or relatively balanced in small-scale breedings No. 1, No. 2 and No. 3. The measured values of egg weight were largely balanced in small-scale breeding No. 4. The values of the egg weight in this small-scale breeding were statistically significant (p <0.05) higher compared to the values of the egg weight of small- scale breedings No. 1, No. 2, and No. 3. Conclusions of the research and the knowledge published in scientific journals are not uniform as regards the impact of factors on eggshell quality.

Huber-Eicher and Sebö (2001) took the view that they showed a higher weight of eggs and their egg components, which were in a negative correlation with the stocking intensity (r = -0.27, p <0.01). The authors pointed out that if laying hens produced more eggs under industrial conditions, the lower the egg weight was recorded. At the end of their investigation, the authors concluded that laying hens that were kept under organic farming conditions, they laid eggs which were generally heavier due to the lower production intensity.

Eggshell weight

Average eggshell weight in individual small-scale breedings is given in Figure 4. Statistically, evaluation of eggshell weight in individual small-scale breedings is given in Table 2.

Egg colour is also an important factor in egg production, in our case brown. Colour shell of eggs can affect consumer choice due to regional or national cultural preferences for different colours, directly affecting eggs' production **(Wei and Bitgood, 1990; Joseph et al., 1999)**.

Thus, the determination of egg colour and eggshell strength is of importance. The average weight of eggshell was found to be either the same or relatively balanced in small-scale breedings No. 1, No. 2 and No. 3. The measured values of eggshell weight were largely balanced in small-scale breeding No. 4. The values of the eggshell weight in this small-scale breeding were statistically significant (p <0.05) higher, compared to the values of the eggshell weight of small-scale breedings No. 1, No. 2 and No. 3. Conclusions of the research and the knowledge published in scientific journals is not uniform as regards the impact of factors on egg shell quality.

Authors **Monira et al. (2003); Alsobayel et al. (2003)** and **Anderson et al. (2004)** agreed in a statement that the quality of the eggshell is sufficiently affected by the genotype and age of laying hens. In characterizing the effect of genotype on egg shell quality, the authors emphasize the significance of genotypic differences in egg weight and shell quality.

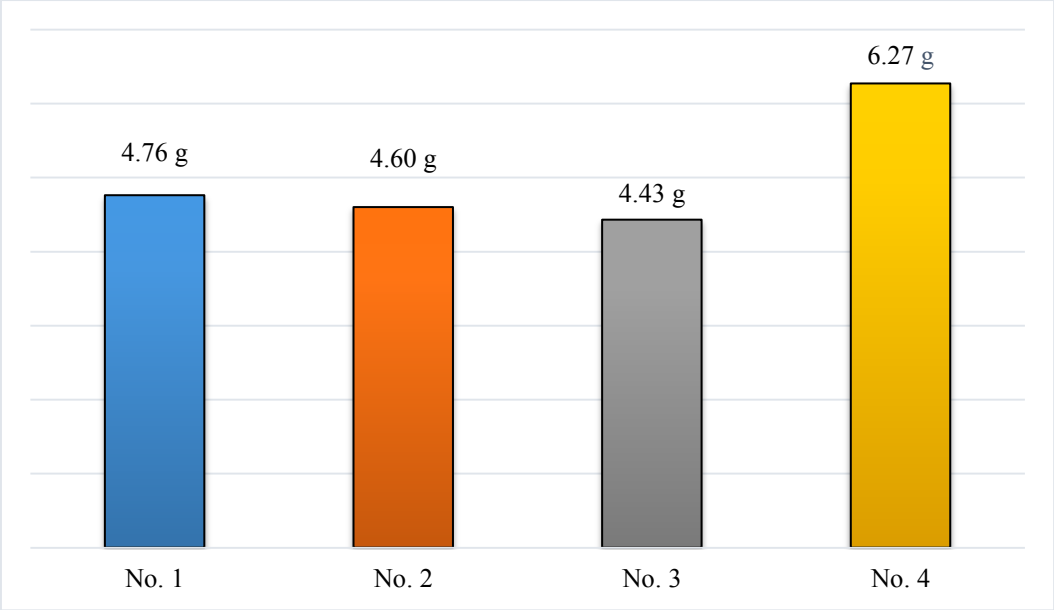

Figure 4 Average eggshell weight in individual small-scale breedings No. 1, 2, 3 and 4; g.

Table 2 Statistically evaluation of egg weight in individual small-scale breedings No. 1, 2, 3 and 4.

Small-scale breeding	F-test 40.47^{+++}				Scheffe´s test		
	n	*SD*	c_v, %	R, g	No. 2	No. 3	No. 4
No. 1	20	0.65	13.58	3.82 – 5.82	-	-	+
No. 2	20	0.74	16.11	2.67 – 5.60		-	+
No. 3	20	0.55	12.40	3.29 – 5.27			+
No. 4	20	0.39	6.24	5.48 – 6.72			

Note: n – multiplicity; *SD* – standard deviation; c_v – coefficient of variation; R – variation range as the difference between the smallest and the largest value of the data distribution; +++: statistically significant difference among group means by analysis of variance (p <0.001); +: statistically significant difference among groups by Sheffe´s test (p <0.05); –: no statistically significant difference among groups by Sheffe´s test (p >0.05).

Such contradictory aspects may also be related to ensuring that laying hens are kept in line with their needs. Therefore, in our research, we focused on characterizing the laying hens and compared them with four small-scale breeders in Slovakia with a focus on selected indicators of the table eggs. Avian eggshells are commonly used in studies focusing on bioindication and environmental monitoring **(Lam et al., 2005; Ayas et al., 2008; Kim and Oh, 2014; Khademi et al., 2015; Simonetti et al., 2015)**.

Shell thickness in the equatorial plane of egg

The average thickness in the equatorial plane of egg in individual small-scale breedings is given in Figure 5.

Statistically evaluation of shell thickness in equatorial plane of egg is given in Table 3.

The average thickness in equatorial plane of egg was found to be either the same or relatively balanced in small-scale breedings No. 1, No. 2 and No. 3. The measured values in the three parts of the equatorial plane of egg were largely balanced in small-scale breeding No. 4. The values of the equatorial plane of the egg in this small-scale breeding were statistically significant (p <0.05) higher compared to the values of the equatorial planes of egg of small- scale breedings No. 1, No. 2 and No. 3

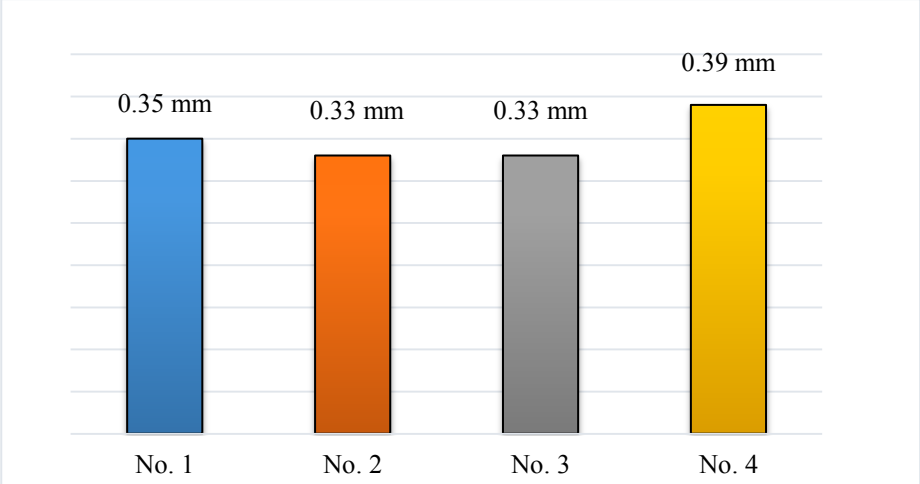

Figure 5 in equatorial plane of egg in individual small-scale breedings No. 1, 2, 3 and 4; mm.

Table 3 Statistically evaluation of shell thickness in equatorial plane of egg in individual small-scale breedings No. 1, 2, 3 and 4.

Small-scale breeding	F-test 10.44^{+++}				Scheffe´s test		
	n	*SD*	c_v, %	R, g	No. 2	No. 3	No. 4
No. 1	20	0.04	11.92	0.28 – 0.44	-	-	+
No. 2	20	0.04	12.23	0.24 – 0.39		-	+
No. 3	20	0.04	10.82	0.27 – 0.39			+
No. 4	20	0.03	7.96	0.31 – 0.43			

Note: n – multiplicity; *SD* – standard deviation; c_v – coefficient of variation; R – variation range as the difference between the smallest and the largest value of the data distribution; +++: statistically significant difference among group means by analysis of variance (p <0.001); +: statistically significant difference among groups by Sheffe´s test (p <0.05); –: no statistically significant difference among groups by Sheffe´s test (p >0.05).

Table 4 Correlation relation (r) between indicators of the eggs in small scale breedings No. 1, 2, 3 and 4, and statistically significant difference between the two variables.

Indicator of egg	S-C B	Eggshell weight				Shell thickness in equatorial plane of egg			
		No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4
Egg weight	No. 1	0.46^{+}				0.17^{-}			
	No. 2		0.18^{-}				-0.16^{-}		
	No. 3			0.42^{-}				-0.11^{-}	
	No. 4				0.53^{+}				0.21^{-}
Eggshell weight	No. 1					0.89^{+++}			
	No. 2						0.83^{+++}		
	No. 3							0.80^{+++}	
	No. 4								0.74^{++}

Note: **S-C B** – Small-scale breeding; numeric value – value r; $^{+++}$: statistically significant difference between the two variables (p <0.001); $^{+}$: statistically significant difference between the two variables (p <0.05); $^{-}$: no statistically very highly significant difference between the two variables (p >0.05).

Correlation relation between egg indicators in small-scale breedings

Correlation relation between indicators of the eggs in small scale breedings and statistically significant difference between the two variables are given in Table 4. Middle, a positive linear relation (small-scale breeding No. 1 and at the lower limit a strong positive linear relation (small-scale breeding No. 4) was found between egg weight and eggshell weight, statistically significant (p <0.05). A very strong relationship in all small-scale breedings (p <0.01, p <0.001) was found between eggshell weight and shell thickness in the equatorial plane of the egg.

Shell thickness in individual parts of the equatorial plane of the egg in small-scale breedings

Average shell thickness in individual parts of the equatorial plane of the egg in small-scale breedings is given in Figure 6. A statistically significant difference between the two variables is given in Table 5. The average thickness in equatorial planes 1, 2, and 3 of the egg was found to be either the same or relatively balanced in small-scale breedings 1, No. 2, and No. 3. The measured values in the three parts of the equatorial plane of egg were largely balanced in small-scale breeding No. 4. The values of the equatorial planes of the egg in this small-scale breeding were statistically significant (p <0.05) higher compared to the values of the equatorial planes of the egg of small- scale breedings No. 1, No. 2, and No. 3.

The eggshell is a natural protection for the egg, and thus it is significant to get a high value of eggshell strength **(Bain, 1990)**.

The eggshell strength, reflecting the resistance ability to

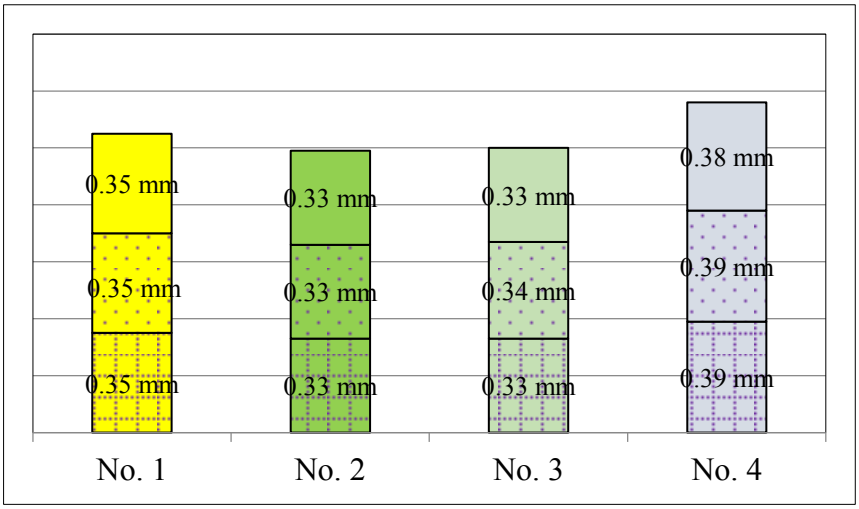

Figure 6 Average shell thickness in individual parts of the equatorial plane of the egg according to small-scale breedings No. 1, 2, 3, and 4, mm.

Table 5 Statistically evaluation of shell thickness in individual parts of the equatorial plane of the egg according to small-scale breedings No. 1, 2, 3 and 4.

Small-scale breeding		n	*SD*	c_v, %	R, mm	Scheffe´s test		
						No. 2	No. 3	No. 4
F-test 10.32^{+++}	Equatorial plane 1							
No. 1		20	0.04	11.65	0.29 – 0.44	-	-	+
No. 2		20	0.04	12.28	0.25 – 0.40		-	+
No. 3		20	0.04	10.73	0.26 – 0.39			+
No. 4		20	0.03	8.26	0.31 – 0.43			
F-test 10.32^{+++}	Equatorial plane 2							
No. 1		20	0.04	11.84	0.28 – 0.44	-	-	+
No. 2		20	0.04	12.67	0.23 – 0.39		-	+
No. 3		20	0.03	10.15	0.27 – 0.40			+
No. 4		20	0.03	7.12	0.32 – 0.43			
F-test 10.32^{+++}	Equatorial plane 3							
No. 1		20	0.04	11.94	0.28 – 0.44	-	-	+
No. 2		20	0.04	12.59	0.23 – 0.39		-	+
No. 3		20	0.04	11.46	0.26 – 0.40			+
No. 4		20	0.03	7.93	0.31 – 0.43			

Note: n – multiplicity; SD – standard deviation; c_v – coefficient of variation; R – variation range as the difference between the smallest and the largest value of the data distribution; +++: statistically significant difference among group means by analysis of variance (p <0.001); +: statistically significant difference among groups by Sheffe´s test (p <0.05); –: no statistically significant difference among groups by Sheffe´s test (p >0.05).

damage, can protect eggs when they are in collecting, packaging, storage, and transportation. It can be found the higher the eggshell strength, the stronger the resistance to damage. Cracked eggs can finally cause economic loss in two ways, one is that they cannot be sold at a high price, another is cracked eggs may raise the risk of bacterial contamination to intact eggs, which can even produce food quality and safety problems **(Bain, 2005; Mertens et al., 2006; Li, Dhakal and Peng, 2012)**.

Correlation relation between shell thicknesses in individual parts of the equatorial plane of the egg according to small-scale breedings

Correlation relation between shell thickness in individual parts of the equatorial plane of the egg according to small-scale breedings and statistically significant difference between the two variables are given in Table 6.

In shell thickness between individual parts of the equatorial plane of the egg in small-scale breedings, No. 1, No. 2, No. 3, and No. 4, an almost perfect positive linear relation was found, statistically very highly significant (p <0.001).

Correlation relation between shell thicknesses in individual parts of the equatorial plane of the egg together for all small-scale breedings

Correlation relation between shell thickness in individual parts of the equatorial plane of the egg together for all small-scale breedings and statistically significant difference between the two variables is given in Table 7.

Almost perfect positive linear relation, statistically very high significant (p <0.001), was found in the shell thickness between the individual parts of the equatorial plane of the egg in all examined small-scale breedings together.

Contamination and damage of table eggs

The percentage and number of eggs with damaged egg surface but also deformed and contaminated on the eggshell are given in Table 8.

Table eggs obtained from a small-scale breeder were subjected to an assessment of the hygiene aspect of the breeding environment. Table eggs can be considered as naturally packaged food. The eggshell serves to contain the egg contents. It is also the first barrier against bacterial penetration and must be free from defects in the order to optimize the safety of human consumption **(Mabe et al., 2003)**.

We found that table eggs were contaminated with blood (from 5 to 45%) and dropping (from 20 to 60%). We found sediments, pigment dots and calcium deposits on the surface of table eggs. Also table eggs from two farms had a deformed shape.

Solomon (2010) reported that the coated eggshell, it is common surface defect observed on the eggshell. There are observed additional calcium deposits or extra-cuticular coverings and possibly reflects the timing and magnitude of the stress or disturbance experienced by the flock. It is commonly observed an incidence of this defect of 1% and could be caused by the age of the laying hens, often younger flocks coming into production **(Coutts and Graham, 2007)**.

Table 6 Correlation relation between shell thickness in individual parts of the equatorial plane of the egg according to small-scale breedings No. 1, 2, 3 and 4, and statistically significant difference between the two variables.

Indicator of shellegg	S-C B	Shell thickness in equatorial plane of egg 2				Shell thickness in equatorial plane of egg 3			
		No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4
Shell thickness in equatorial plane of egg 1	No. 1	0.97^{+++}				0.99^{+++}			
	No. 2		0.97^{+++}				0.99^{+++}		
	No. 3			0.95^{+++}				0.97^{+++}	
	No. 4				0.96^{+++}				0.97^{+++}
Shell thickness in equatorial plane of egg 2	No. 1					0.99^{+++}			
	No. 2						0.99^{+++}		
	No. 3							0.97^{+++}	
	No. 4								0.99^{++}

Note: **S-C B** – Small-scale breeding; numeric value – value r; +++: statistically significant difference between the two variables (p <0.001); +: statistically significant difference between the two variables (p <0.05); –: no statistically very highly significant difference between the two variables (p >0.05).

Table 7 Correlation relation between shell thickness in individual parts of the equatorial plane of the egg together for all small-scale breedings and statistically significant difference between the two variables and statistically significant difference between the two variables.

Indicator of shellegg	Shell thickness in equatorial plane of egg 2	Shell thickness in equatorial plane of egg 3
Shell thickness in equatorial plane of egg 1	0.97^{+++}	0.98^{+++}
Shell thickness in equatorial plane of egg 2		0.99^{+++}

Note: numeric value – value r; +++: statistically significant difference between the two variables (p <0.001).

Table 8 The percentage and number of eggs with damaged egg surface but also deformed and contaminated on the egg shell (n = 20 for each small-scale breeding).

Smal-scale breeding	No. 1		No. 2		No. 3		No. 4	
Indicator	pcs	%	pcs	%	pcs	%	pcs	%
Blood stains	2	10	9	45	8	40	1	5
Dropping stains	9	45	6	30	12	60	4	20
Pigment dots	9	45	7	35	13	65	9	45
Sediments – other	5	25	6	30	8	40	3	15
Calcium deposits	2	10	0	0	4	20	3	15
Small bumps	2	10	1	5	2	10	1	5
Deformed egg shape	1	5	0	0	0	0	3	15

Blood stains

Calcium deposits

Small bumps

Figure 7 Contamination and damage of table eggs in small-scale breedings.

These damaged table eggs, but also deformed and contaminated on the surface, are related both to internal factors and to external factors, which the farmer can influence and take measures to improve laying hens living conditions. **Hincke et al. (2000)** reported that there are multiple factors affecting eggshell quality like the genetics of the hen, nutrition, and management of feed intake, disease, and environment challenge, and also equipment insult.

A decline in eggshell quality is detected as hens approach the end of a laying period **(Mazzuco and Hester, 2005)**. In this way, the condition of the eggshell at the oviposition time can influence the incidence of shell breakage. An interesting insight presents **Alves et al. (2007)**. When the laying hens are raised in conditions of greater thermal comfort, it can promote eggshell quality and decrease egg losses through cracks.

Hulzebosch (2004) states in his study that eggshell plays a very important role. It must form a good barrier against the intrusion of microorganisms into the internal egg content. Many research results confirm increased microbial contamination in alternative breeding compared to laying hens.

In alternative breeding, laying hens lay eggs more extensively outside the nest, into the litter. Such eggs show excessive contamination of their surface. Such eggs have a damaged shell, which can lead to the deterioration of the internal content of the egg and its contamination. There are two ways in which the contents of a table egg can be infected. It can be infected by an endogenous route and an exogenous route. **Engelmaierová, Tůmová and Charvátová (2010)** state that endogenous contamination occurs through sick laying hens, which affect the egg through the bloodstream. Exogenous contamination of table eggs is caused by microorganisms that are in the outdoor environment.

Görner and Valík (2004) in the study point out that there is a large number of spores on the surface of the eggshell that are highly permeable to air. The cuticle is an outer layer whose main function is to prevent microorganisms from entering the egg. When the eggs are brought into contact with the air, the cuticle is drawn into the spores, changing its shape, which results in deformation, causing penetration of the microorganisms through the shell into the internal contents of the egg. An important protective barrier is also represented by the membrane membranes. Their fibrous structure acts as a filter.

Their protective properties are associated with the chemical action of lysozyme and ovotransferrin. Microorganisms, by means of proteolytic enzymes, disrupt the membranes and penetrate the whites. The main role of egg white is to protect the egg yolk from contamination. Gram-positive bacteria are affected by egg white due to their antimicrobial and bactericidal effects. Egg yolk that has no antimicrobial properties is the perfect breeding ground for the reproduction of microorganisms. If a high incidence of contamination has been observed on the surface of the eggshell, there is a higher risk of contaminants penetrating the egg content. **Křepelka (2012)** points out that contaminated eggs are a major problem in terms of consumer protection, which must be constantly eliminated. In most cases, gram-negative bacteria, e.g. *Pseudomonas* spp., *Alcaligenes* spp. *Salmonella enteritidis* and *Escherichia coli* and gram-positive bacteria such as e.g. *Bacillus* spp. and *Staphylococcus*.

CONCLUSION

Table eggs from small-scale breeding are preferred by the consumer. Literary sources are poor and inconsistent in the knowledge of laying hens breeding conditions in small-scale breeding, and the quality and safety of table eggs. Because the food consumer likes table eggs from small-scale breeders, we have researched this issue. Based on the obtained and statistically evaluated results there were formulated the following conclusion:

(a) The average egg weight was equalized in three small-scale breedings and the fourth small-scale breeding was significantly higher. Higher egg weight is related to the higher age of laying hens.

(b) The average eggshell weight and shell thickness in the equatorial plane of the egg was balanced in three small-scale breedings and the fourth small-scale breeding was significantly higher. Higher eggshell weight may be related to improved conditions in breeding hygiene, as confirmed by the results of the investigation of contamination and damage of table eggs. These differences may also be related to nutrition.

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PERCEPTION OF SENSORY ATTRIBUTES AND MARKETING TOOLS OF SELECTED MILK BRANDS

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ABSTRACT
The research has shown that in the last 5,000 years the human evolution has made the greatest leap in the human history. As a result of these changes, thanks to relatively recent discovery of a specific gene, even adult Europeans can digest milk. In their nutritional guidelines based on the scientific evidence, the official health organizations and institutions recommend drinking milk and eating low-fat dairy products such as yoghurts or cheese. The human body absorbs the necessary amount of calcium in the easiest form from cow's milk. Calcium is the essential element not only for healthy bones and teeth but it is also the important factor for the transmission of nerve impulses, it supports heart activity, helps reduce high blood pressure and "bad" LDL cholesterol levels and alleviate allergic reactions. It has impact on proper blood clotting, affects sperm mobility, helps prevent arthritis or can also contribute to better sleep. Milk is a valuable food not only for calcium content but also for selenium which slows down the aging process and contributes to the protection of the immune system. Acid dairy products prevent the digestive disorders, protect against gastrointestinal infections and improve skin condition. Despite all these positive aspects the consumption of milk, as well as dairy products, has the downward trend in Slovakia. In the last decade, the consumption was at a historical minimum and below the recommended annual dose, which is 91 liters of milk per person. Therefore, the main objective of this research paper was to examine the quality of milk produced by two selected competing companies and identify the various factors affecting consumers´ decision-making process when purchasing milk and dairy products. The attention was concentrated on the sensory attributes (colour, appearance, smell, taste and quality) and marketing tools (brand, packaging, label and price). The primary data were obtained by the questionnaire survey, which was conducted in the Slovak Republic with 284 respondents. For a deeper analysis, the data were evaluated by the statistical methods. Based on the results of the blind testing it was determined that even though the quality of monitored milk is the same, the respondents prefer the sensory attributes of Rajo semi-skimmed milk. When it comes to brand, packaging, label and price Tami semi-skimmed milk also lagged behind.

Keywords: milk; consumers; Slovakia; Rajo semi-skimmed milk, Tami semi-skimmed milk

INTRODUCTION
Milk is a rich source of nutrients and energy. It is one of the complex foods and it can be called as a "superfood" because it contains the numerous nutrients. Most of the ingredients that are present in milk do not act in isolation but in interactions. Milk can contribute significantly to the intake of substances such as magnesium, selenium, riboflavin, vitamin B12 and pantothenic acid. The bioavailability of some nutrients in milk, such as calcium, is very high compared to other foodstuffs, and therefore, drinking milk in childhood is of a great importance in promoting growth and healthy bone development. Milk sugar, called lactose, participates in the formation of brain cells. Among other things, milk has a low sodium content and contains up to 87% water what makes it suitable for maintaining a daily drinking regime. In addition, milk does not contain the substances that inhibit the bioavailability of minerals such as phytates or oxalates. The recent research has highlighted the beneficial effects of milk on the health of the subjects **(Weaver, 2014)**, body weight **(Wang et al., 2014)**, and obesity related diseases including type 2 diabetes and cardiovascular disease **(Markey et al., 2014; O'Connor et al., 2014).** Therefore, the role of milk and dairy products has been the subject of debate in the recent years, both in the scientific and popular-scientific literature.

However, the opinions on the issue of drinking milk are divided into two contradictory streams today. According to **Norton (2015)** the most common reasons why people who can drink cow's milk tend to choose alternative sources of milk (plant based drinks) or even completely exclude milk and dairy products from their diet are following:

- milk quality – industrial packaged milk has the different taste and consistency and also lower nutritional value,
- the cows´ life quality (moral aspect) – life of cows on farms and in large-scale production

enterprises is often distinguished by suffering, because the animals are not treated properly,
- the impact of milk on human health – milk increases the pH level of organism and causes acidification. Then it uses calcium as one of the most effective means to achieve alkalinity when trying to balance pH,
- humans are "mammals" – they should drink just human breast milk during breastfeeding,
- veganism – not consuming meat or any other animal products.

On the positive side, the actual research done by **(Golian et al., 2019)** marks these upper mentioned reasons as myths and scientifically refuses them.

Milk and dairy products have been a traditional food of the people in our country since the time immemorial. This fact was confirmed in the publication written by authors **Podolák and Mjartan (1974)** who wrote about the ancestors of the Slovaks as breeders, since the history of dairy production has been developed for around more than 100 years. Milk along with cheese and yoghurt are three most commonly consumed dairy products. Most countries have the quantitative recommendations that usually range from 2 to 3 servings or cups of milk or yoghurt, or sometimes the equivalent serving of cheese a day. In 1989, according to **Kubicová, Kádeková and Dobák (2014)**, Czechoslovakia consumed nearly 80 kg of milk per capita but during 17 years (1995 – 2012) milk consumption as well as dairy products per capita had a downward trend. In the last decade, consumption **(VUEPP, 2019)** hit the historical lows (Figure 1) and it was below the recommended annual dose, which according to **Chlebo, Šrámková and Harasník (2009)** is 91 liters per person. The Slovak Republic has considerable reserves in this indicator, hence it is important to examine quality and marketing tools which can persuade people **(Chandon, and Wansink, 2012)** to buy and subsequently consume milk.

Scientific hypothesis

We assumed that:
- logos are easier to remember for people who know the chosen brands,
- there are differences in assessing of the selected categories related to packaging,
- the pricing strategies have the different effects.

H_0: there is not a correlation between awareness of brand and memorability of a logo.
H_1: there is a correlation between awareness of brand and memorability of a logo.

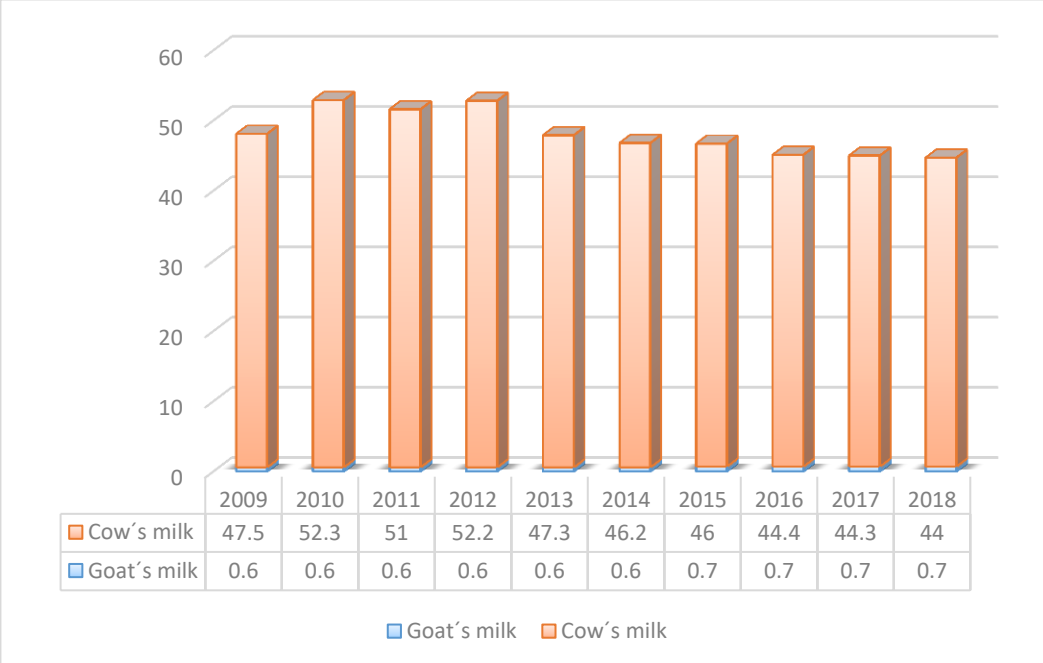
H_0: there are no differences in assessing of the selected categories.
H_1: there are differences in assessing of the selected categories.

H_0: The pricing strategies are identical.
H_1: The pricing strategies are different.

MATERIAL AND METHODOLOGY

The main objective of this research paper was to examine the quality of milk produced by two selected competing companies and identify various factors affecting consumers´ decision-making process when purchasing products. The focus was targeted at the sensory attributes (colour, appearance, smell, taste and quality) and marketing tools (brand, packaging, label and price).

The first part of this paper is a theoretical overview that allowed a better understanding of the topic. The secondary information was obtained by using the method of collecting data from publicly available information, the internet sources, literary sources of domestic and foreign authors, as well as articles by experts on consumer behavior on milk market.



	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Cow´s milk	47.5	52.3	51	52.2	47.3	46.2	46	44.4	44.3	44
Goat´s milk	0.6	0.6	0.6	0.6	0.6	0.6	0.7	0.7	0.7	0.7

Figure 1 Total consumption of drinking milk in Slovakia in liters per capita.

Since many authors are currently devoted to this topic, we have sorted the data by using selection and processed it into the appropriate form for this paper via deduction, analysis and synthesis. This theoretical overview became the basis for the realization of questionnaire surveys which served as a source of primary information and was supplemented with a blind testing. The aim of the questionnaire survey was to determine consumer habits in the process of purchasing and consumption of milk and sensory preferences of milk. The respondents were addressed in person and they filled in the electronical questionnaire form which was created in the Google Forms. Firstly, people were asked 8 classification questions and then 22 factual questions, which were primarily open or semi-open, with pre-selected options for answering. The data were collected from 284 respondents in the period from 4 February 2019 to 19 October 2019.

In total, 135 men and 149 women participated in the questionnaire survey. Maintaining representativeness was also important when categorizing respondents by age. The respondents of all ages were approached in order to copy the structure of the population of the Slovak Republic. The respondents aged from 36 to 45 (20.42%) represented the largest share. The same percentage of respondents (15.85%) identified the options 'from 46 to 55 years' and 'from 56 to 65 years'. 2% more (17.96%) were over the age of 66 years. The lowest representation was in the age category over 18 to 26 years. Only 32 respondents were included, which represents 11.27% of the sample in terms of percentage. The remaining 18.66% belonged to the age group from 26 to 35 years. The questionnaire form also included the question asking about the highest achieved education. 7 respondents (2.46%) completed only the elementary education. The secondary education without a school-leaving exam was marked by 16.20% and the similar education which ended with school-leaving exam by 53.87%. In the case of the higher education, the respondents had the choice of three alternatives. 11.27% completed university education of bachelor´s degree, 15.14% of master´s degree and 1.06% the doctoral university education. The fourth classification question was devoted to the economic activity of the respondents. The smallest groups were economically inactive respondents without any job (3.52%) and mothers in the maternity leave (4.93%). They were followed by 'self-employed or entrepreneur' with 8.45%. The category 'student' was the third biggest group with 15.85% and the second biggest group was that of pensioners (22.89%).

The majority of respondents (44.37%) were employed, who were also asked to write what position they were working at. Since this additional question was open, we received the different answers. The total income of household depended on the economic activity of the respondents. The most numerous groups earned from 1,001 to 1,500 € (30.28%) and from 501 to 1,000 € (26.06%). In terms of place of residence, 54.93% came from villages and 45.07% identified with the option city. All of these respondents were inhabitants of Slovakia.

Statistical analysis

Every question in the questionnaire was described verbally and some of them were also evaluated graphically. For a better understanding of the correlation, the methods of qualitative statistics were also used. The statistical evaluation program XLStat 2019.3.1 Build 60464 was used by Addinsoft to realise the following tests:

- Chi-Square Goodness of Fit Test,
- Chi-Square Test of Independence
- McNemar´s test,
- Friedman´s test,
- Nemenyi's procedure.

Traditionally, the following approach was applied to test the hypotheses:

- formulation of hypotheses H_0 and H_1 and determination of significance level α,
- after evaluating the distribution of relevant test statistics, the definition of the critical area at the significance level α,
- calculation the value of the test statistics based on empiric data,
- evaluation whether the value of the test statistics is from a critical area of the test. If it is, reject H_0 and if it is not, accept H_0.

RESULTS AND DISCUSSION

The consumers are familiar with Rajo and Tami brands in the selected market. Up to 97.54% of them knew the Rajo brand and 85.56% Tami. This high representation can be due to the fact that customers can find milk or other dairy products from at least one of these brands in almost every grocery store in Slovakia.

Majority of the respondents liked and were satisfied with the design of the Rajo and Tami logos. Only 13.03% are dissatisfied with the design of Rajo and 19.36% with the Tami design. In the following open question this minority stated that the Rajo logo is too simple, lacks colour and the used red color is very aggressive. On the other hand, the Tami logo is old-fashioned and many people have described it as "retro". In addition to the above-mentioned suggestions, the respondents who disliked logos agreed on an overall redesign that would change the font type and size, and in their opinion, it also lacked the slogan and image of something associated with milk (according to many respondents, it would be appropriate to add an image of cows).

According to the results of the questionnaire survey, the logos of selected brands are relatively easy to remember. Only 3.87% have trouble remembering the Rajo logo and 13.38% the Tami logo. Marketing and advertising agency **Navigation Advertising (2018)** claims that a logo must be easy to read, recognize, remember and reproduce. We tested this question using chi-square test for independence:

H_0: there is not a correlation between awareness of brand and memorability of logo.
H_1: there is a correlation between awareness of brand and memorability of logo.

Rajo: *p*-value = <0.0001
Tami: *p*-value = <0.0001

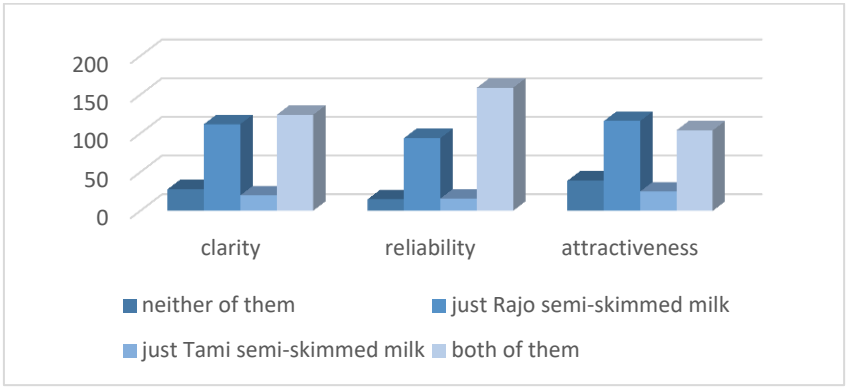


Figure 2 Assessment of packaging.

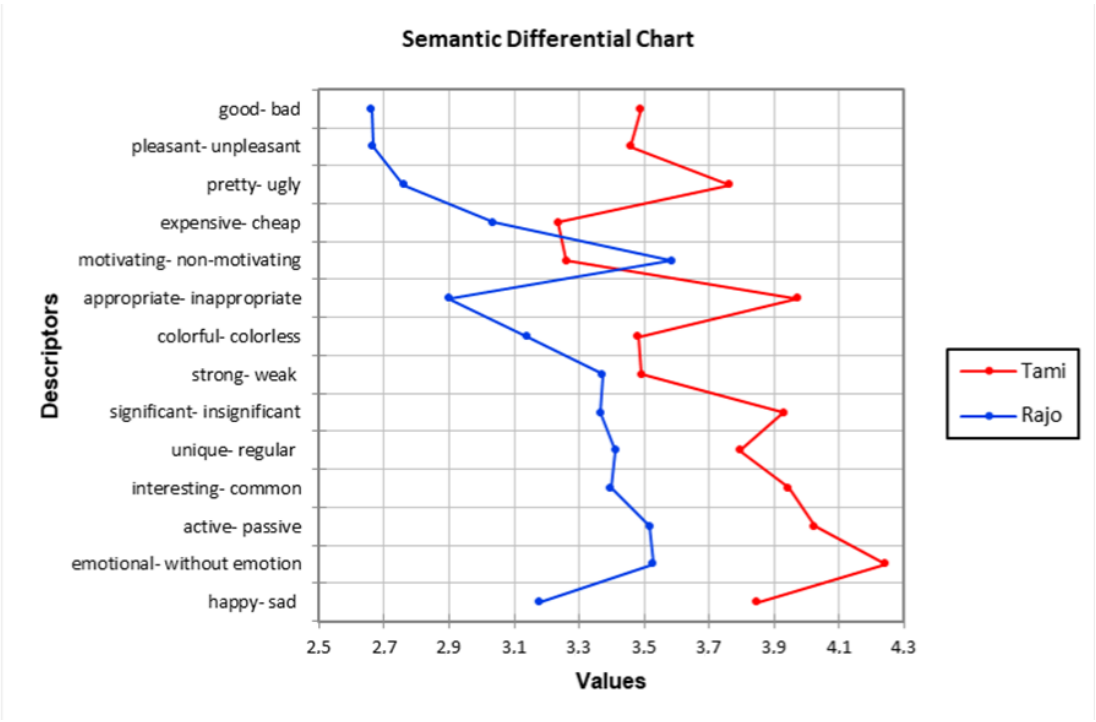


Figure 3 Assessment of selected categories.

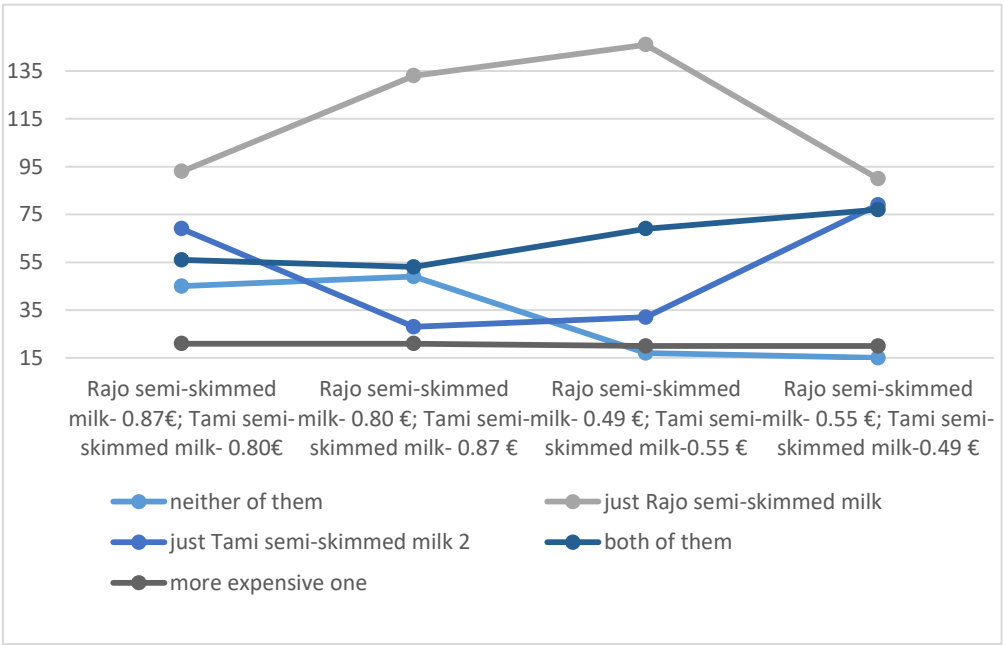


Figure 4 Selection of milk based on price.

Both tests showed that there is a correlation between awareness of brands and memorability of logos because *p*-value was smaller than alpha and we accepted the alternative hypotheses.

Approximately 60% of milk purchases are made based on brand. 21.83% of respondents targeted exclusively at Rajo and 9.51% at Tami Packaging.

Packaging

Three following questions of the questionnaire form were related to the expression of the respondents´ opinion on the clarity, readability and attractiveness of competing milk packaging, because when the right visual features and the right communication with the different segments of consumers are used, the promoted product can be successful in the market and consumers can be persuaded to make beneficial choices as well **(Rybanská, Nagyová and Tkáč, 2019)**. From the graph (Figure 2), it is evident that the direct comparison of chosen milk brands referring to clarity of packaging, more consumers inclined to Rajo (39.43%) and Tami was chosen just by 7.04%. In terms of readability, the situation is exactly the same, as Rajo was marked by 33.09%, while Tami just by 5.63%. The results of the packaging attractiveness are similar to the clarity.

In general, the respondents assessed both packaging and the ease to read, having appealed labels containing information useful to the customer, and last but not least, they appreciated the usage of an attractive blue-white color combination, which is also best suited for milk packaging according to **Clark (2016)** and **Rybanská et al. (2019)**. Moreover, blue is a favourite colour of 57% men and 35% of women, which also plays in favor of these types of packaging **(Ciotti, 2020)**. The majority agreed that "both types of packaging are attractive, even though they present milk differently and each of them addresses a different group of customers". The research conducted by **Horská et al. (2018)** showed that Slovak consumers prefer traditional packages connected with Slovak customs and traditions associated with nature, farming, home production and high quality **(Rybanská, Nagyová and Horská, 2019)** to more modern packages of milk.

The semantic differential was also used to further assessment of the appearance of the packaging, which consisted of a 7-degree scale by which individuals evaluated 14 bipolar pairs of adjectives. The adjectives by which the respondents expressed their attitude were categorized into dimensions: evaluation, strengths and activities. The dimension of evaluation is most important because it reflects the impression of packaging. The second dimension (strengths) represents the energy charge and the last dimension (activity dimension) focuses on dynamics assessing. The picture shows that Rajo was evaluated by respondents as a better one in almost all categories except for the motivation, where Tami won (Figure 3).

The Friedman´s test was used for each packaging to test the statistically proven difference in assessing each category:

H_0: there are no differences in assessing of the selected categories.
H_1: there are differences in assessing of the selected categories.

Rajo: *p*-value = <0.0001
Tami: *p*-value = <0.0001

The level alpha significance (0.05) is greater than the calculated *p*-values in both cases, so we confirm the alternative hypothesis, and the results of Nemenyi's procedure confirmed these differences too, since the variables were categorized to different groups where A was rated as the best (Table 1 and Table 2).

Price

According to **Kurajdová and Táborecká-Petrovičová (2015)** and **Kubicová, Predanocyová and Kádeková (2019)**, there is a relationship between the price factor and consumer behavior applied by Slovak consumers when purchasing milk, so for this reason the perception of the different price combinations of selected products and purchase decisions made by them were examined. If both types of milk were sold for the same price, more respondents (75.35%) tended to purchase Rajo semi-skimmed milk and Tami semi-skimmed milk was chosen by only 24.45%.

Subsequently, 4 situations were discussed (Figure 4). Two of them were real: Rajo semi-skimmed milk – 0.87 € and the pricing strategies are different in effect, Tami semi-skimmed milk – 0.80 €, when the current prices, which were observed on 21th January 2019 at Tesco were used and the discount prices (Rajo semi-skimmed milk – 0.49 € and Tami semi-skimmed milk – 0.55 €), and two simulated situations (Rajo semi-skimmed milk – 0.80 € and Tami semi-skimmed milk – 0.87 €; Rajo semi-skimmed milk – 0.55 € and Tami semi-skimmed milk – 0.49 €) when milk prices were exchanged. Approximately 7.39% of the respondents would buy another more expensive type of milk. Almost 50 respondents would not have bought any of the products during the assessment of current prices, but after a price reduction these numbers fell to one fourth. Quite the opposite situation occurred in the answer “I would buy both products" because when the prices changed to reduced ones, the numbers of respondents increased by almost a half. Looking at the exclusive purchase of the individual brands, Tami's deep drop of 59.42% was seen in the second situation (Rajo milk – 0.80 €; Tami milk – 0.87 €), when more questioned people would choose Rajo brand. A further increase in Rajo to 52.76% of the sample was due to a stock price lower than that of Tami milk. When Rajo was more expensive than Tami (in the last simulated situation), Rajo was also chosen by 14 customers more than Tami. We can assume that some consumers are price-sensitive so even those who have previously chosen Rajo would prefer to buy more affordable milk.

The effectiveness of price levels was tested by McNemar´s test, where the pairs of situations (normal price level with simulated replaced price level) were compared.

H_0: The pricing strategies are identical.
H_1: The pricing strategies are different.

p-value = <0.0001
alpha = 0.01

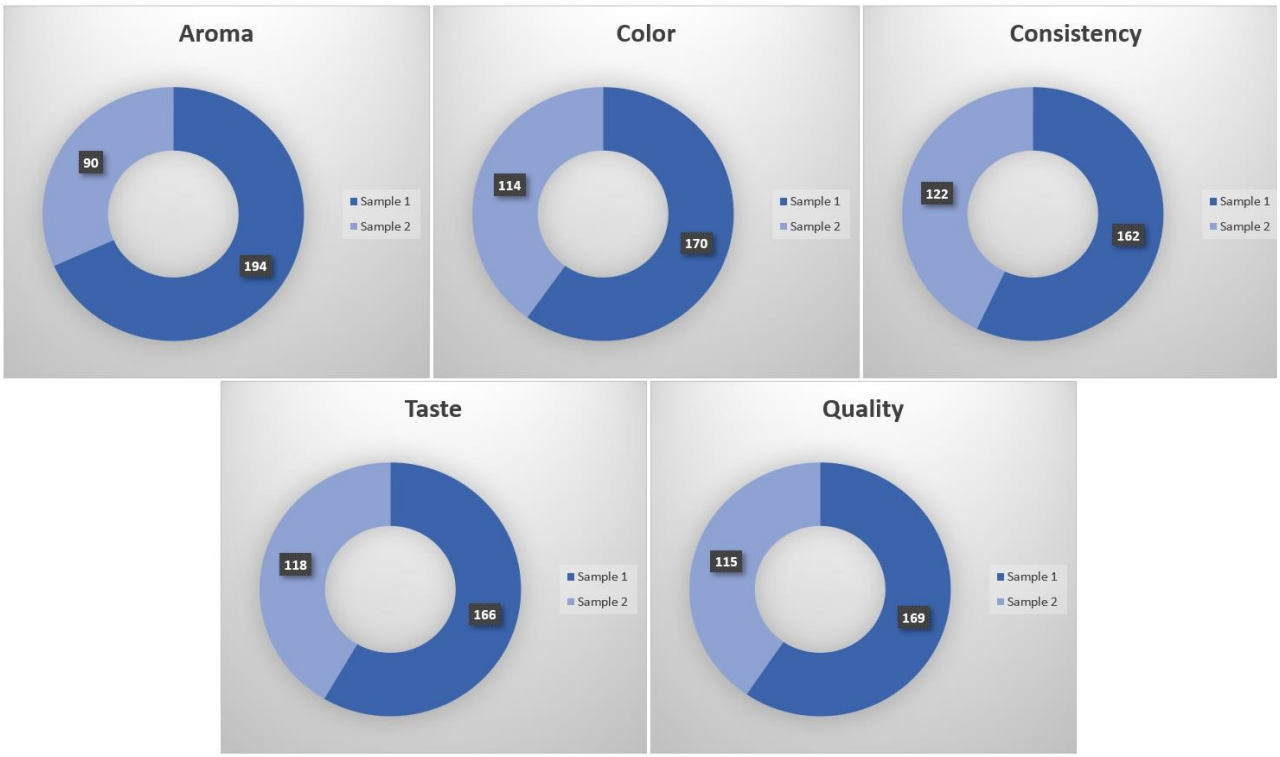


Figure 5 Results of blind testing.

Table 1 Classification of selected adjectives when evaluating the package of Rajo milk.

Rajo	Frequency	Sum of ranks	Mean of ranks	Groups					
good – bad	284	1,588.500	5.593	A					
pleasant – unpleasant	284	1,597.500	5.625	A					
pretty – ugly	284	1,671.000	5.884	A					
appropriate – inappropriate	284	1,800.000	6.338	A	B				
colorful – colorless	284	2,049.500	7.217		B	C			
happy – sad	284	2,105.500	7.414		B	C	D		
significant – insignificant	284	2,269.000	7.989			C	D		
interesting – common	284	2,269.500	7.991			C	D		
unique – regular	284	2,283.000	8.039			C	D		
strong – weak	284	2,297.500	8.090			C	D		
emotional – without emotion	284	2,349.500	8.273			C	D		
motivating – non-motivating	284	2,415.500	8.505				D	E	
active – passive	284	2,416.500	8.509				D	E	
expensive – cheap	284	2,707.500	9.533					E	

Table 2 Classification of selected adjectives when evaluating the package of Tami milk.

Tami	Frequency	Sum of ranks	Mean of ranks	Groups					
pleasant – unpleasant	284	1,807.500	6.364	a					
expensive – cheap	284	1,821.500	6.414	a	b				
good – bad	284	1,869.000	6.581	a	b	c			
appropriate – inappropriate	284	1,882.500	6.629	a	b	c	d		
colorful – colorless	284	1,885.500	6.639	a	b	c	d		
pretty – ugly	284	2,146.000	7.556		b	c	d	e	
significant – insignificant	284	2,179.500	7.674			c	d	e	f
happy – sad	284	2,210.000	7.782				d	e	f
unique – regular	284	2,262.000	7.965					e	f
motivating – non-motivating	284	2,264.500	7.974					e	f
interesting – common	284	2,292.500	8.072					e	f
strong – weak	284	2,300.000	8.099					e	f
active – passive	284	2,394.500	8.431					e	f
emotional – without emotion	284	2,505.000	8.820						f

Table 3 Nutrition facts of chosen milks per 100 mL.

Nutrition facts per 100 ml			
Rajo semi-skimmed milk		**Tami semi-skimmed milk**	
Energy	200 kJ/48 kcal	Energy	194 kJ/46 kcal
Fat	1.5 g	Fat	1.5 g
of which Saturates	0.9 g	of which Saturates	0.9 g
Carbohydrates	4.9 g	Carbohydrates	4.9 g
of which Sugars	4.9 g	of which Sugars	4.7 g
Protein	3.4 g	Protein	3.2 g
Salt	0.15 g	Salt	0.2 g
Calcium	125 mg	-	

The results of both tests showed that with 99% probability the pricing strategies have statistically proven the different efficiencies (*p*-value is less than 0,0001), so we accepte the alternative hypothesis that there is the difference in efficiency of pricing strategies. In the first pair of situations, the Rajo brand is preferred and in the second pair the respondents inclined to purchasing a cheaper type of milk.

More than three quarters of the research sample (78.17%) is convinced that the price level at which selected products are sold in stores is adequate to their quality. According to the remaining 62 respondents, the price does not reflect the quality of neither examined product. **Andocsová (2019)** explains this difference in perception of prices as a result of the economic activity of respondents.

The respondents who marked the price as inadequate in the previous question were asked for an acceptable price level. 12.90% of respondents indicated the possibility of over 80 cents, paradoxically, as Rajo milk is sold for 0.87 € and Tami for 0.80 €, so these 8 respondents are most likely to be poorly informed about the current prices at which the selected products are realized on the market. The corresponding price of 71 to 80 cents sees 2.47%, from 61 to 70 cents 7.42% and 26.86% indicated the possibility of 51 to 60 cents. According to the majority of 36.04%, the common price should be the stock price, and 22.26% are in favor of not paying more than 40 cents for these products.

Blind testing

The authors **Kurajdová, Táborecká-Petrovičová and Nedelová (2019)**, **Krivošíková et al. (2019)** and **Kubicová, Predanocyová and Kádeková (2019)** emphasize that consumers living in Slovakia derive the quality of milk based on the real experience with its freshness and sensory perceptions. For this reason a blind testing was also used. It involved all 284 respondents who received 2 samples of room temperature milk in the same translucent plastic cups labeled 1 (Rajo semi-skimmed milk) and 2 (Tami semi-skimmed milk). It is important to highlight that the respondents did not know which number contained which brand during the testing. This information was provided after the testing was finished.

From the perspective of the conscious perception of the individual sensory attributes (Figure 5), which are the most important for consumers **(Krivošíková, Géci and Nagyová, 2019)**, up to 68% of the respondents preferred Rajo milk based on aroma. 60% of them selected Rajo milk semi-skimmed according to colour too, and it was also chosen by the majority because of its consistency (57%). Following this question, the respondents were asked to taste the submitted samples. Similarly, to the results of the previous questions, Rajo (58%) was repeatedly considered as tastier and higher quality product. An interesting finding was that most of the respondents, regardless whether they liked Rajo more or not, could clearly identify this brand of milk. After a personal interview, we found out that according to them it has a specific taste and smell. An unusual finding that although Tami is a big competitor of Rajo, it lags in the monitored milk properties, as the respondents chose the sample number 1 in all categories.

As Rajo semi-skimmed milk won the blind testing in all categories, we wondered whether there was a difference in nutrition facts per 100 mL of Rajo semi-skimmed milk and Tami semi-skimmed milk (which were also compared in the blind test). Table 3 shows that these two brands of milk are almost the same, nevertheless, Rajo is preferred.

CONCLUSION

The results of the questionnaire survey, the conducted blind testing and microbiological testing showed the following: the majority of respondents liked both logos (Rajo 86.97 % and Tami 80.64 %) and they also thought that they are easy to remember. Packagings were evaluated as clear, readable and attractive, but some respondents would make minor changes. When both types of milk are sold at full price, the respondents prefer Rajo semi-skimmed milk but when it comes to price reductions, they tend to be price-sensitive and purchase milk whichever is available for a lower price **(Merlo, 2015)**. The respondents preferred Rajo semi-skimmed milk in all categories and they identified it as the one with higher quality even though based on microbiological testing there was no difference between the examined samples. All of our assumptions were also confirmed by the statistical methods.

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THE NOVA SYSTEM AND ULTRA-PROCESSED FOODS IN RELATION TO CONSUMER DECISION-MAKING IN FOODS CHOICE

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ABSTRACT

The purpose of the study was to investigate strawberry yogurt according to the NOVA food classification system. The object of research were strawberry yogurts from 6 different manufacturers, which are commonly available to food consumers, were randomly selected to investigate food samples under the NOVA food classification system. Based on this food, we present a methodology for assessing food safety with the application of the procedure. At the same time, we justify the classified food to Group 4, i.e. one of the 4 groups according to the NOVA system of food classification on a scientific basis, knowledge from the scientific literature. The evaluated results of the labeling of strawberry yogurt from various producers indicate that they are all classified as ultra-processed foods. Their characteristic feature is that they are industrial products with five or more, and usually many, items. Strawberry yogurt samples from various manufacturers evaluated contained 7 to 12 specific items that are not basic raw material, i.e. unprocessed or minimally processed food of Group 1.

Keywords: NOVA food classification system; strawberry yogurt; specific item; methodology; ultra-processed food

INTRODUCTION

The term ultra-processed foods come from the NOVA classification scheme, which divides foods into four groups:

Group 1: *unprocessed or „minimally processed" foods*, including fruit, vegetables, and meat. Foods in this group may be processed in a manner that does not add other ingredients. They may be cooked, ground, dried, or frozen.

Group 2: *processed culinary additives*; they are substances obtained directly from foods of group 1 or nature by processes such as pressing, refining, grinding, milling, and spray-drying; they are processed food items, including sugar, salt, and oils, when the items of this group are combined, for example by the production of salty butter, this product remains in this group.

Group 3: *processed foods*, represents the combination of unprocessed or minimally processed foods with processed culinary additives; i.e. Group 1 and Group 2. These are bread, wine, and canned vegetables. Additives are allowed provided they preserve the original characteristics of the food, such as ascorbic acid added to preserved fruit to prevent browning.

Group 4: *ultra-processed foods* do not have a strict definition, but the NOVA system points to some characteristics. They usually have five or more additives. They can be aggressively sold and highly profitable.

The risk of ultra-processed foods of Group 4 is from „substances which are not normally used in culinary preparations and additives intended to imitation the sensory properties of Group 1 foodstuffs or culinary preparations thereof or to mask (conceal) undesirable sensory properties of the finished product" **(Monteiro et al., 2016)**.

The risk of ultra-processed food of group 4 is from "substances not normally used in culinary preparations and additives intended to imitate the sensory properties of Group 1 foodstuffs or culinary preparations from these foods or mask (conceal) undesirable sensory properties of the final product" **(Monteiro et al., 2018)**.

Hall et al. (2019) followed hospitalized adult patients who received ultra-processed and unprocessed food every day for 14 days. The diet was compared for energy intake, sugar, fat, fiber, and macroscopic minerals. Ad libitum intake was 2,093.4 $kJ.day^{-1}$ (500 kcal) more for ultra-processed foods compared to unprocessed food. The body weight changes of these respondents were highly correlated with dietary differences in energy intake (see Figure 1).

The ultra-processed products listed in the second row are not variants of the food and meal mentioned above. They are composed of industrial food substances but contain little or no unprocessed food. They are unhealthy for their character and should be grouped. We should avoid these foods **(Monteiro et al., 2016)**.

The definition of ultra-processed foods has been gradually developed and improved over the years 2009 to

2017 **(Monteiro, 2009; Juul et al., 2018**; **Monteiro et al., 2010**; **Moubarac et al., 2014**; **Monteiro et al., 2010**; **Monteiro et al., 2016; Costa Louzada et al., 2015c; Martínez Steele et al., 2016)**.

In the publication of the team of **Monteiro et al. (2016)** the food groups 1, 2, 3, and 4 of the NOVA system are characterized in detail.

We focused on Group 4 foods, i.e. ultra-processed foods and drinks in our article.

Group 4 NOVA food products are food and beverage products that are fully processed. These are industrially manufactured products with five or more, and usually many, items. Additives often include those also used in processed foods such as sugar, oils, fats, salt, antioxidants, stabilizers, and preservatives.

Additives found only in ultra-processed products include those not commonly used in culinary preparations and additives. The purpose of the additives in ultra-processed foods is to imitate the sensory properties of Group 1 foods or culinary preparations from these foods or to mask the undesirable sensory properties of the final product. Foods in Group 1 are proportional or absent in ultra-processed products.

Substances that are only found in ultra-processed products are some directly extracted from foods such as casein, lactose, whey, and gluten and some derived from further processing of food ingredients such as hydrogenated or interesterified oils (fatty acid exchange in the triacylglycerol structure), hydrolyzed proteins, soy protein isolate, maltodextrin, invert sugar and high fructose corn syrup.

Food additives found in ultra-processed products include

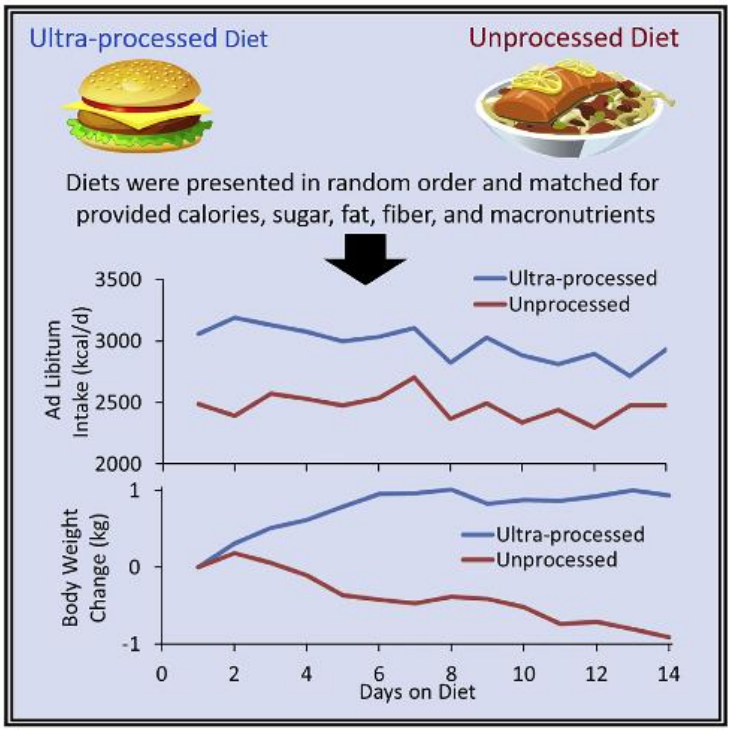

Figure 1 High correlation between intake of different energy content from ultra-processed and unprocessed foods ad libitum and body weight **(Hall et al., 2019)**.

Figure 2 Unprocessed (1st row) and ultra-processed foods (the 2nd row) **(Monteiro et al., 2016)**. Note: The first row: fruit; grains and legumes; stew with beans and vegetables; water. The second row: fruit flavored popsicles; breakfast cereals; reconstituted meat product; non-alcoholic drinks.

coloring, flavor enhancers, sugar-free sweeteners, and processing aids such as carbonation, firming, volume expansion and leveling, antifoams, anti-caking and glazing agents (e.g. palm kernel oil), emulsifiers, sequestrants, and humectants.

Some industrial processes without domestic equivalents are used to produce ultra-processed products such as extrusion and molding and pre-frying.

The main purpose of industrial ultra-processing is to produce products that are ready to be consumed, drunk, or heated and which can replace unprocessed or minimally processed foods that are naturally ready to be eaten, such as fruit and nuts, milk and water, and freshly prepared beverages and meals. Common features of ultra-processed products are excessive tastiness, sophisticated and attractive packaging, multimedia, and other aggressive marketing for children and adolescents, health claims, high profitability, and multinational corporations.

Examples of typical ultra-processed products are carbonated beverages; sweet or savory snacks; ice cream, chocolate, confectionery; meat packaged pastries; margarine and spreads; biscuits, pastries, cakes, and cake mixes; breakfast cereals, energy drinks; dairy drinks, „fruit' yogurts" and „fruit drinks"; cocoa drinks; meat extracts, including chicken and „instant" sauce; infant formulas, milk after milking, other products for infants; powdered or „enriched" meal substitutes; many ready-to-cook products, including pre-made cakes and pasta and pizza; poultry and fish „nuggets" and „sticks", sausages, hamburgers, hot dogs and other reconstituted meat products and powdered and packaged „instant" soups, noodles and desserts.

Where products produced exclusively from foodstuffs of Group 1 or 3 also contains so-called „food" cosmetic or sensory enhancing additives, such as plain yogurt with added fruit containing artificial sweeteners and breads with added emulsifiers, are classified here in Group 4. Where alcoholic beverages are identified as foods, these produced by fermentation of the foods of Group 1, followed by distillation of the resulting alcohol, such as whiskey, gin, rum, vodka, are classified in Group 4.

Advice based on NOVA knowledge on ultra-processed foods in the area of nutrition in relation to good human health, and the accumulated evidence of their effects in national dietary habits and welfare, it is relatively simple but significant to avoid consuming them. This issue of ultra-processed foods is elaborated e.g. in the official dietary recommendations for Brazilian citizens, where the issue is addressed not only in research but also in politics **(Brazilian Ministry of Health, 2014)**.

The importance of industrial processing in terms of methods and uses of food additives or produced by modern food science and technology in terms of food properties and the state of food in relation to human health, it is still undervalued. This state of so-called relative neglect is contained in reports and other documents that include recommendations on diet, epidemiological studies, and in policies and strategies aimed at improving nutrition and health of the population. In the first half of the last century, the guidelines on eating, they contained most of the meals that were a combination of foods with culinary additives and consumed as meals as such. But the second half of the last century is characterized by food packaging, labeling, and instructions for preparing for eating or heating. These „fast" or „profitable" foods became more and more preferred, they have become more important in the dietary models of high-income countries **(Monteiro et al., 2018).**

Monteiro (2009) published an opinion claiming that the scope and purpose of food processing had changed worldwide. These changes are leading to a harmful global food system and a pandemic of obesity and other nutritional causes of chronic non-communicable diseases.

The NOVA system classification scheme according to **Monteiro et al. (2016)**, which contains four groups of foods, according to the extent and purpose of the processing to which they are subject, includes physical, biological, and chemical processes. These processes occur after the separation of natural foods and before their consumption or use in the preparation of meals. The methods used in the culinary preparation of foods in household kitchens, restaurants, public catering in general, including the disposal of wastes from cooking meals, spices, and blending various foods, the NOVA classification scheme is not taken into account.

The purpose of the study was to investigate strawberry yogurt according to the NOVA food classification system.

Scientific hypothesis

Scientific hypothesis: Based on the evaluation of strawberry yogurt samples from different manufacturers, that these industrial products will contain items, which will be a characteristic attribute and count for inclusion in ultra-processed foods.

MATERIAL AND METHODOLOGY

Object of study

Strawberry yogurts, which are commonly available to food consumers, were randomly selected to investigate food samples under the NOVA food classification system. Based on this food, we present a methodology for assessing food safety with the application of the procedure. At the same time, we justify the classified food to Group 4, i.e. one of the 4 groups according to the NOVA system of food classification on a scientific basis, knowledge from the scientific literature.

Characterization of strawberry yogurt samples

Strawberry yogurt samples from 6 different manufacturers were investigated based on the NOVA food classification system according to **Monteiro et al. (2016)**. These authors have included fruit yogurts among the Group 4 foods, which are ultra-processed.

Method

Strawberry yogurt samples were assessment based on composition, i.e. labeling on the packaging. Each food ingredient that is characterized by Group 4 foods has been identified by a number in the order in which the strawberry yogurt items were listed. For assessment of strawberry yogurt, 6 pcs samples, were available. Each sample originated from a different manufacturer. Initially, the composition items for each strawberry yogurt sample indicated on the packaging were identified by the order

number. The final number of the sample composition item characterized the sum of strawberry yogurt items.

The NOVA food classification system according to **Monteiro et al. (2016)** contains fruit yogurts in Group 4 foods, i.e. in ultra-processed foods characterized by 5 or more specific items in the composition.

RESULTS AND DISCUSSION

The evaluated results of strawberry yogurt are given in Table 1.

The table shows the composition of strawberry yogurt from 6 different manufacturers. Given the risks associated with their consumption, which follow from the literature review, our effort was to propose a methodology for their identification, i.e. a simple way that would be understandable for food consumers. The food consumer selects the food itself when buying the food. He decides which food to choose from.

Several factors influence his choice, but one of the most important is a health-related choice. We aimed to assess risks from processed foods, including ultra-processed foods and facilitating decisions in choosing foods for consumers.

This is the initial work, which is closely linked to the tracing of food labels by food consumers, which is already a common part of food purchases.

The present study presents a broader link between consumer decisions in food choice and public health. Even now, in many cases, the food consumer is already deciding on the choice of food based on its composition. The scientific basis for this decision is based on the Nova system, which is presented in this article.

The evaluated results of the labeling of strawberry yogurt from various producers indicate that they are all classified as ultra-processed foods. Their characteristic feature is that they are industrial products with five or more, and usually many, items. Additives are used in them as in processed foods (sugar, stabilizers, and preservatives). Other items have been found in them, which are typical only for ultra-processed products and which are not commonly used in culinary preparations and culinary additives (such as aromas, coloring, acidity regulators, etc.).

Research from recent years confirms data that medicines are being processed, which are now the main shaping force, which has become a global food system and is a key determinant of eating methods and possible health and well-being **(Monteiro, 2009; Ludwig, 2011; Stuckler et al., 2012; Moodie et al., 2013)**.

Analysis of survey data confirmed findings from the consumption of ultra-processed foods **(Costa Louzada et al., 2015a, Costa Louzada et al., 2015b; Steele et al., 2015; Cespedes and Hu, 2015).**

Food classification according to the NOVA system it is clear, useful, understandable, and easy to use **(Monteiro et al., 2016)**.

According to **Rico-Campà et al. (2019)** the methodology of the NOVA system classification of foodstuffs was criticized, but according to these authors, there is no better alternative. It is also easy to use for reporting and reproducible and therefore beneficial to public health. Also, it is the best known and most commonly used classification of ultra-processed foods in epidemiological studies.

Therefore, these authors used the classification a NOVA system for identifying four different food groups by stage of processing. Their interest was in the fourth group under the NOVA system, which included ultra-processed food and beverages that tend to be nutritionally unbalanced due to several industrial process operations.

These foods are economically advantageous because the shelf life and hence the sale of these foods increases, but the quality of the nutritional value decreases. Ultra-processed foods are characterized by high energy, low fiber, and microscopic minerals and high added or free sugars, sodium, saturated fats, and chemical food additives **(Moubarac et al., 2013)**.

Intake of ready-to-eat, ready-to-drinks, ready-to-eat products has increased significantly in all countries, irrespective of economic level over the last two decades. This trend may have contributed to a worldwide increase in total cancer **(Fiolet et al., 2018)**, dyslipidemia **(Rauber et al., 2015)**, obesity **(Rauber et al., 2016)**, and hypertension **(Mendonça et al., 2017)**.

Srour et al. (2019) report in their publication results from the prospective cohort study NutriNet-Santé, during 5.2-year research and conclude that the results could explain some of the scientific hypotheses established in the research of ultra-processed foods in relation to cardiovascular diseases.

The first interpretation: Ultra-processed foods generally have worse nutritional quality than unprocessed or minimally processed foods because they tend to be richer in sodium, energy, fat, and sugar, and poorer in fiber **(Luiten et al., 2016; Moubarac et al., 2017; Cediel et al., 2018)**; they are also associated with a higher glycemic reaction **(Costa Louzada et al., 2015a)**.

Sugar sweeteners can delay the initiation of the inner satiety signal, which can lead to excessive energy intake **(DiMeglio and Mattes, 2000)**.

In a prospective NutriNet-Santé cohort study, participants included in the high consumption group of ultra-processed foods had lower fruit and vegetable intake. It is well known that a high intake of fruit and vegetables (food Group 1) together with respect for a healthy diet is beneficial for the prevention of cardiovascular diseases (high level of evidence) **(Mozaffarian, 2016)**.

The second interpretation: Refers to a wide range of additives in ultra-processed foods. Although the highest permitted levels protect consumers from the adverse effects of individual substances in certain foods **(World Health Organization, 2018)**, the effect of the cumulative effect of intake of all foods used and the potential or interaction effect remains largely unknown, unexplained **(Srour et al., 2019)**.

For some, from approximately 350 different permitted food additives in Europe, several adverse effects on cardiovascular disease have been identified in animal experiments or cell model studies.

Also, emulsifiers often found in ultra-processed foods, particularly carboxymethylcellulose and polysorbate **(Santé Publique France DREES, 2018)**, demonstrated potential effects of low degree in inducing inflammation and obesity or metabolic syndrome in mice **(Chassaing et al., 2015)**.

Table 1 Strawberry yoghurt samples from various manufacturers.

Sample	Basic raw material	Flavoring ingredient	Yoghurt culture
No. 1	**cream**	1 sugar 2 strawberry **thickening:** 3 maize starch, 4 strawberry concentrate 5 black carrot concentrate 6 aroma	**7 yes**
No. 2	**cream**	1 sugar 2 strawberries 3 glucose-fructose syrup 4 modified corn starch 5 water 6 aromas **thickening:** 7 pectins **coloring:** 8 carmines, 9 acidity regulators 10 citric acid 11 sodium citrates	**12 yes**
No. 3	**milk**	1 sugar 2 glucose-fructose syrup 3 strawberry puree 4 strawberry juice **stabilizer:** 5 modified starch 6 aromas **coloring**: 7 carmine 8 concentrate juice from beetroot **acidity regulators:** 9 citric acid 10 milk proteins	
No. 4	**milk, cream**	1 sugar 2 strawberries 3 water **thickening:** 4 modified corn starch 5 strawberry aroma identical to natural 6 natural coloring carmine **acidity regulators:** 7 sodium citrate	**8 yes** and 9 probiotic culture
No. 5	**milk**	1 strawberries 2 sugar **stabilizer:** 3 pectin 4 lemon juice 5 milk protein	**6 yes** and 7 Bifidobacterium, 8 *Lactobacillus acidophilus*
No. 6	**milk**	1 pieces of strawberries 2 water 3 sugar **coloring concentrates:** 4 of black carrot 5 of carrot 6 natural aroma 7 concentrated skimmed milk 8 sugar 9 milk proteins	**10 yes** and 11 „Bifidus“

Note: – the strawberry yoghurts are commonly available for food consumers and they are from different manufacturers; – the composition of the individual strawberry yogurt samples is used from their labelling on the package; – the number for the strawberry yogurt ingredient in the table indicates the order of the ingredients of the composition with the final number of ingredients.

Ultra-processed foods may be contaminated with contact materials (suspected of migrating from the packaging), including bisphenol A in some plastic packaging, which the European Chemicals Agency considers as „Substance of Very High Concern" **(European Chemical Agency, 2016)**. This substance is associated with an increased risk of cardiometabolic sequelae (especially hypertension and coronary artery disease) in a recent meta-analysis **(Rancière et al., 2015)**.

CONCLUSION

The NOVA food classification system is a simple and effective way of assessing food based on its division into 4 groups. Food group characterization is important. This way of assessment foods and dividing them into any of the 4 groups is important for the consumer to make their choices. Based on the assessment of strawberry yogurts that have been the subject of our research, it follows that, according to the characterization of the NOVA food classification system, they are included in Group 4, among ultra-processed foods. Strawberry yogurt samples from various manufacturers evaluated contained 7 to 12 specific items that are not basic raw material, i.e. unprocessed or minimally processed food of Group 1.

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SENSORY EVALUATION AND ACCEPTANCE OF FOOD MADE OF EDIBLE INSECTS

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ABSTRACT
This paper discusses the sensory analysis of food enriched with selected edible insect species which are labelled as a novel food – house cricket (*Acheta domesticus*) and mealworm (*Tenebrio molitor*). Energy bars of two different compositions with the addition of cricket flour and puff pastry bars sprinkled with the whole roasted mealworm larvae were evaluated by consumer tests performed via a questionnaire survey. Respondents represented both men and women in equal numbers and included consumers with the experience of the sensory analysis and edible insects to ensure accurate results. Sensory analyses revealed that respondents associated their tastes with already known flavors (salty, sweet, bitter, fish, French fries, chicken, and mushrooms). The most common answer from respondents was a salty taste, followed by a sweet taste. There were also unusual comparisons such as pine seeds. Consumers' positive attitude to these energy bars has been registered which shows that the Czech consumer accepts edible insects in a suitable form as a novel food and possible part of the food basket.

Keywords: edible insect; *Tenebrio molitor*; *Acheta domesticus*; sensory analysis

INTRODUCTION
Entomophagy is a practice of edible insect consumption **(Imathiu, 2020)**. The origin of this word comes from Greece, where ἔντομον éntomon means "insects" and φᾰγεῖν phagein is translated as "to eat" **(Kouřimská and Adámková, 2016)**.

Sensory properties comprise an important criterion for edible insect consumption **(Borkovcová et al., 2009; Adámek et al., 2017)**. In western countries, entomophagy is often associated with dirt and poverty, thus being considered unacceptable **(Looy, Dunkel and Wood, 2014)**. Many people disregard insects and they neglect its high nutritive value **(Siemianowska et al., 2013)**. Existing significant prejudices closely connect with a disgust factor representing a major repulsive force that is formed in the child age before 6 or 7 years. In contrast, an edible insect has been consumed in other cultures in Africa, Latin America, Asia **(Hanboonsong, 2010; Raheem et al., 2019)** and Australia **(Siemianowska et al., 2013)**. There are approximately two billion people in 113 countries consuming edible insects **(Lucas et al., 2020)**. Recently, around 2000 species of edible insects have been registered **(Megido et al., 2018)**. Countries with a common entomophagy practice include Zimbabwe, Japan **(Raheem et al., 2019)**, Thailand with more than 150 edible insect species **(Imathiu, 2020)**, and Mexico with approximately 550 species of edible insect consumed **(Siemianowska et al., 2013)**. Consuming edible insect is also supported by the fossils **(Kouřimská and Adámková, 2016)**. Technologies of insect mass rearing have been accepted in some countries, such as Holland, Denmark, Belgium, Kenya, and Vietnam **(Imathiu, 2020)**. In China, where entomophagy is practiced for more than 3,000 years **(Lucas et al., 2020)**, edible insect also gains the role of a food ingredient and is seen as a suitable alternative to further kinds of meat **(Kröncke et al., 2018)**.

The insect may be consumed indirectly in a form of extracts or products, such as honey, wax, pollen, and dyes **(Lucas et al., 2020)**.

The potential of edible insects has been growing due to positive environmental impacts **(Dossey, Morales-Ramos, and Rojas, 2016)**. The rearing of edible insects requires less water and space if compared to other livestock. What is more, the amount of produced greenhouse gas is considerably lower (**Lucas et al., 2020**). Another advantage of growing an edible insect is its high reproduction rate **(Imathiu, 2020)**. Apart from these significant benefits, edible insect as a part of a balanced diet could be a potential source of adequate food supplies due to its high feed conversion rate **(Belluco et al., 2013; Van Huis et al., 2013)**. Furthermore, according to studies, edible insect species provide great nutritional values as they are an excellent source of protein, fat, vitamins as well as minerals **(MacEvilly, 2000; Van Huis et al., 2013)**. Several

conditions can change the nutritional value of edible insects, such as the environmental conditions or different processes of heat treatment **(De Castro et al., 2018)**. The edible insect could be eaten in a raw form or could be processed by roasting, frying, or boiling **(Imathiu, 2020)**. Interestingly, frying may even improve the sensory quality of insects due to aromatic compounds, attractive colors, crust, and texture. Its hygienic quality may be enhanced by a cooking process thanks to the inactivation of possibly present pathogenic microorganisms **(Megido et al., 2018)**. Unfortunately, heating processes could affect nutritional values as well **(Montowska et al., 2019)** due to the processes including proteolysis, lipolysis, and lipid oxidation. **(Megido et al., 2018)**.

A prediction of a significant increase in edible insects on the global market is expected to reach 710 million dollars **(Roncolini et al., 2020)**. Mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus*) are the species most commonly farmed in Europe. What is more, they are considered to be the most promising in the food and feed industry **(Imathiu, 2020)**.

Crickets (*Acheta domesticus*) contain a substantial amount of all-important constituents – proteins, fats, vitamins, and minerals **(Montowska et al., 2019)**.

Based on a questionnaire created by **Bednářová et al. (2013)**, the mealworm was evaluated as one of the most acceptable species for consumption in the Czech Republic. The field cricket was also assessed positively; however, some respondents dislike the feeling of biting into the soft insect body. Their opinion was influenced by the psychological aspect of considering cricket as the food of the poor. Furthermore, European consumers can be divided into two groups – one group prefers the possibility of consuming food with proper visibility of the larvae, while the latter prefers to consume insects in a hidden form **(Adámek et al., 2018)**. This way, the texture of the insect is not easily perceptible. Edible insects, particularly adult specimens, are partly composed of the exoskeleton causing the crunchiness during the consumption influencing the tactile and auditory effect resulting in, along with the chewing, a pleasant feeling similar to the consumption of pretzels, cookies, or other various durable pastry **(Ramos-Elorduy, 1998)**. A beneficial high content of chitin in the exoskeleton should be emphasized **(Aguilar-Miranda et al., 2002; Mlček et al., 2014)**. However, the human body can digest soft larvae after molting because their exoskeletons are not hardened yet **(Borkovcová et al., 2009)**. For this reason, the mealworm larvae were examined in this study.

The taste of the insect could be very diverse. It depends on many factors, such as the environment and the feed including fruits, vegetables, pastries, potatoes, rice, or grass. During the culinary treatment, insect absorbs different flavors of other ingredients which were proved as early as in 1971 by **Smith et al**. **(1971)**. What is more, if the insect is processed with spices, it will obtain a new flavor and the original taste will be weakened. If it is washed before the consumption, which is not recommended by **Ramos-Elorduy (1998)** due to the food safety point of view **(Bednářová et al., 2010)**, it will provide hardly any flavor since the pheromones on the insect surface are rinsed away. That is why to ensure full and rich taste it is necessary to serve insect alive. Due to the health and safety considerations, it is recommended to allow the insect to starve for at least 12 hours. This precaution is recommended particularly for grasshoppers, caterpillars, and beetle larvae **(Ramos-Elorduy et al., 1997)**.

This work aimed to explore the attitude of the general public towards entomophagy in the Czech Republic employing a questionnaire and to evaluate the sensory properties of selected food enriched with the edible insect.

Scientific hypothesis

Food enriched with edible insects is acceptable for consumers in the Czech Republic.

MATERIAL AND METHODOLOGY

Material for sensory analysis

Two species of edible insects were selected for the sensory analysis – mealworm (*Tenebrio molitor*) and house cricket (*Acheta domesticus*). Specimens of mealworm (*Tenebrio molitor*) were purchased in a pet shop and left to starve for 48 hours, killed with boiling water (100 °C), and dried at 105 °C. Subsequently, the insect was sprinkled on the puff pastry bars which were examined in the sensory analysis (Figure 1).

House cricket (*Acheta domesticus*) was sensory evaluated in a form of cricket flour. The sample of cricket flour was used in energy bars. Respondents were provided with two kinds of bars. Sample A contained dates, cricket flour, pineapple, cashew, coconut, psyllium, and lemon peel. Sample B included dates, cricket flour, cocoa powder, cashew, psyllium, and orange peel. Bars were cut into dices, spiked with toothpicks, and offered to the respondents for evaluation.

Survey methodology

Samples were subjected to the sensory analysis and evaluated using a questionnaire. In the first part of the research, the samples of mealworm (*Tenebrio molitor*) were evaluated focusing on the taste. The short form of the questionnaire, designed especially for the wide public, contained mainly questions about the respondent (age, gender) and the taste of insects. Respondents were asked to specify the taste of the sample. Thirty-two respondents participated in the first survey and 53% of them have already experienced food sensory analysis under the laboratory conditions. 25 respondents participated in the second survey with only 40% having the experience of the food sensory analysis.

Bars containing cricket flour were examined in the second part of the experiment using the third questionnaire. This survey was attended by 42 respondents, of which 59% were women and 40% were men. Participants in the lecture were provided with two samples of bars (samples A and B) and forms to evaluate the taste. The respondents were asked to evaluate the pleasantness of both samples by using an ordinal score scale from 1 (excellent taste) to 5 (annoying taste).

Figure 1 Puff pastry bars sprinkled with mealworm (photo Jan Gajdošík).

Statistical analysis

The data were analyzed using Excel 2013 (Microsoft Corporation, USA) and STATISTICA CZ version 12 (StatSoft, Inc., USA), and the results were expressed by mean ± standard error.

Considering cricket flour bars, the results were evaluated using a paired t-test to analyze differences between the means of the evaluation of samples A and B.

The dependency of the evaluation on the gender was evaluated using the χ^2 test which was applied to map differences of preferences between genders. For this test, a modified table was used with the preference marked as follows:

A = evaluator scored a better rating for the sample A,
B = evaluator scored a better rating for the sample B,
O = both samples gained the same score from the evaluator.

Puff pastry bars sprinkled with the mealworm larvae were evaluated by the percentage of the taste expressed by the respondent to the total number of answers.

RESULTS AND DISCUSSION

Puff pastry bars sprinkled with mealworm

During the first part of the sensory analysis, a questionnaire survey was used to evaluate samples of mealworm on puff pastry bars. In this first survey, the most significant amount of 31% of respondents described the taste as salty. Other respondents described the taste as sweet (almost nutty) (18.8%), which was the second most selected answer. A similar number of respondents tasted it as the whole grain bread (12.5%) and fish (12.5%). A slightly lower number of respondents opted for the taste of the fried potatoes (9.4%). A small percentage of respondents described the taste as similar to pine seeds, chicken, bitter or other. None of the respondents considered the taste like sour, fatback with skin, mushrooms, apples, raw corn caviar, or herring. A percentage representation of individual flavors is shown in Figure 2.

The results of the second part of the survey are depicted in Figure 3. Most respondents agreed on the option of the sweet taste (24%) and whole grain bread taste (24%).

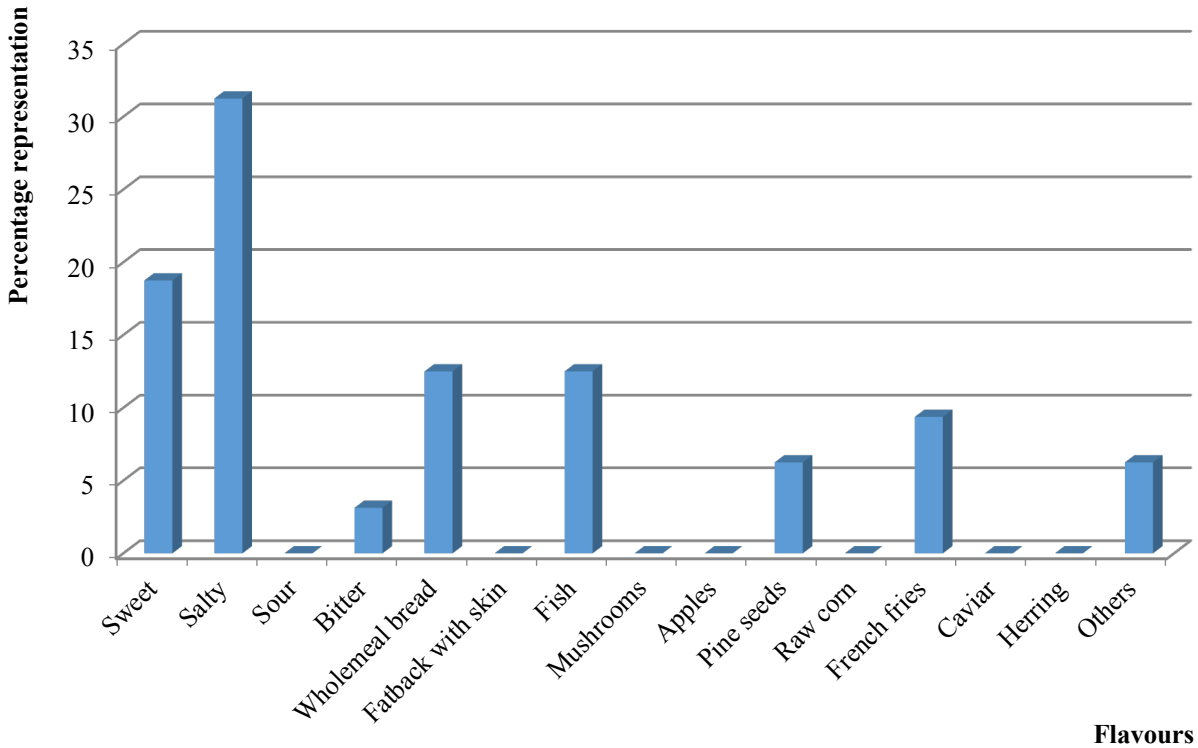

Figure 2 Percentage representation of individual flavours of mealworm samples on puff pastry bars in the survey no.1.

A slightly lower number of respondents (20%) selected salty taste and 16% of respondents described the taste as bitter; the taste was poorly represented in the previous survey. The taste of the pine seeds was selected by 8% of respondents, the option "other" was selected only by one respondent who described the taste as similar to chicken. These results are following the study by **Ramos-Elorduy (1998)** who states that the taste of mealworm (*Tenebrio molitor*) is similar to the taste of the whole grain bread.

There were some similarities in the descriptions of the tastes of both samples – in both cases, the participants selected sweet and salty flavor and the taste of the wholemeal bread and pine seeds. The second sample was characterized by the increase in the frequency of the selection of the sweet and bitter taste and the taste of the wholemeal bread. The flavors of fish and French fries were described only in the first sample. The options for the sour taste and the taste of mushrooms, apples, raw corn, or caviar were not selected by any participant during the analyses.

The edible insect is a highly popular delicacy, especially for its sensory properties **(Borkovcová et al., 2009)**. The flavor of insects varies from species to species, and it is affected by pheromones, the environment where insects live, and the feed they eat. **Ramos-Elorduy (1998)** stated a range of flavors from fishy to wheat bread in the case of major classes of edible insects, (adopted from **Mishyna, Chen and Benjamin, (2020)**). Generally, mealworms aroma is stated as nutty or whole wheat bread **(Ramos-Elorduy, 1998; Capponi, 2016; Elhassan et al., 2019)**. **Roncolini et al. (2019)** further specify that larvae fed flour or cereal bran have a characteristic sweet, almost nutty flavor, and a nutty, cocoa smell. In contrast, larvae which were as fed grass crickets have a strong crustacean-like, cooked legume-like, and earthy aroma.

The sweet flavor is in accordance with the results of this study because the producer fed the larvae with cereal bran. It has been assumed that the high value of the salty flavor is due to the dough on which the larvae were sprinkled.

Energy bars enriched with cricket flour

In the sensory evaluation of energy bars with the addition of cricket flour, two samples were examined. Sample A containing dates, cricket flour, pineapple, cashew, coconut, psyllium, and lemon peel, and sample B containing dates, cricket flour, cocoa powder, cashew, psyllium, and orange peel. There was no statistical difference between the results of each sample ($p > 0.05$). Furthermore, the results have shown a positive evaluation of both samples which approaches the overall average score 2 as can be seen in Figure 4.

Elhassan et al. (2019) described the flavor of crickets as umami, popcorn, chicken, mild, or creamy. On the other hand, **Capponi (2016)** characterized the flavor of cricket as fishy.

According to the results, the influence of gender on the sample evaluation has not been statistically significant ($p > 0.05$) for any of the samples. **Table 1** shows a small but statistically not significant difference ($p > 0.05$) as women preferred sample A from B, while men preferred B. The reason may be the content of cocoa powder in sample B which women like more than men, e.g. in the form of chocolate candies **(Kozelová et al., 2014)**.

In Hungary people who plan to eat less meat or who are looking for new food choice expect to eat edible insects as a substitute. In the study was also noticed that women have bigger neophobia than men **(Gere et al., 2017)**.

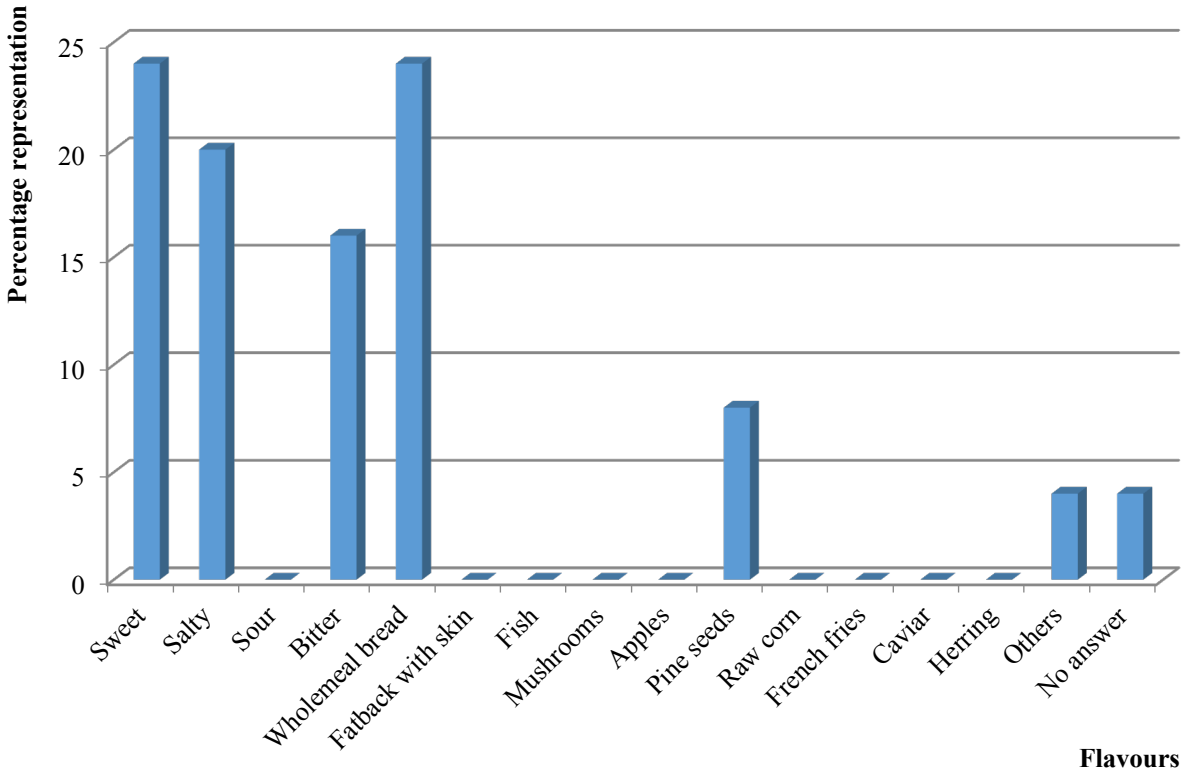

Figure 3 Percentage representation of individual flavours of for mealworm samples on puff pastry bars in the survey no. 2.

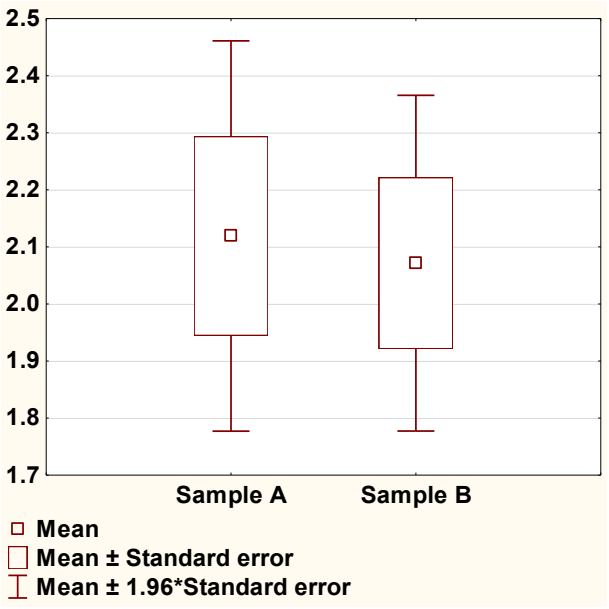

Figure 4 Sensory evaluation of the pleasantness of taste for energy bars enriched with cricket flour in the questionnaire survey no. 3. Note: Sample A – dates, cricket flour, pineapple, cashew, coconut, psyllium and lemon peel; Sample B – dates, cricket flour, cocoa powder, cashew, psyllium and orange peel.

Table 1 Differences of gender preferences for two samples of energy bars enriched with cricket flour in the questionnaire survey no. 3.

Gender	Preferences			
	Sample A (%)	Sample B (%)	Both samples (%)	Total (%)
Woman	40	48	12	100
Man	47	41	12	100
χ2 test	0.21	0.16	0.00	0.38

Note: 1) Preferences: A = better rating for the sample A, B = better rating for the sample B; 2) Critical value of tested criterium χ^2 for significance level 0.05 and 2 degrees of freedom was 5.991.

The study **Megido et al. (2013)**, which was focused on Belgian consumers, found out the people seemed to be willing to eat and cook insects in the future when it is connected with familiar flavors.

The positive result about the consumption of eating insect-containing products in the future is positively accepted by people in Italy and a slightly higher number of males were open to the idea of a product as an alternative source of protein **(Sogari, Menozzi and Mora, 2017)**. In the USA or India are people considering trying to eat some form of insect food and at the same time, men are more inclined to this idea **(Ruby, Rozin and Chan, 2015)**.

On the other hand, in Germany is still a prevalence of skepticism towards the consumption of edible insects. The acceptance is similar between both genders if the insect is in an invisible form **(Orsi et al., 2019)**. In the study, **Hartmann et al. (2015)** were examined the acceptance of edible insect in a comparison between Germany and China without noticeable differences between males and females. A similar study focused on the acceptance of edible insect was the comparison of people from Korea and Ethiopia, where was the acceptance of men higher than women in both studied nations **(Ghosh et al., 2020)**. Another research was done in a Danish college where the respondents were females. Even though in other studies males are more open to eating edible insects than females, in this research 81% of respondents (only females) tasted the mealworms **(Jensen and Lieberoth, 2018)**.

In the study by **Tuccillo et al. (2020)** and **Laureati et al. (2016)** was found that men are more open to entomophagy than women. The same result was mentioned in **Woolf et al. (2019)** where was investigated the opinion of consumption of insect-containing food. Positive willing to trying of insects as food among Dutch consumers were young males, who tried it before **(Tan, van den Berg, and Stieger, 2016)**. **Verbeke (2015)** calculated that the predicted acceptation of edible insects as a substitute for meat is 12.8 % for men and 6.3 % for women.

Megido et al. (2016) examined the acceptance of hybrid insect-based burgers where men were more open to eating burgers that contain edible insects.

The interest of consumption of edible insects in products is being investigated by **Gmuer et al. (2016)**, **Le Goff and Delarue (2017),** or **Hartmann and Siegrist (2016)** where the results were slightly positive. The higher acceptance of edible insects by males when females are mentioned in the study by **Verneau et al. (2016)** where the experiment was held in Denmark and Italy.

Edible insects and consumers in the Czech Republic

During the process of preparation, such as applying the boiling water and cooking edible insects, the original aroma is often removed and the insect acquires the smell and taste of other present ingredients **(Ramos-Elorduy, 1998)**. This could be the reason why the respondents also reported the salty taste of the sticks sprinkled with larvae of mealworms and the hazelnut taste was suppressed. Concerning energy bars enriched with the cricket flour, the flavor of the cricket flour was significantly suppressed by the fruit ingredients. Although the customer in the Czech Republic is currently sufficiently informed about edible insects from festivals, TV shows, or magazine articles, the difference between the perceived tastes of edible insects in the visible and hidden form was obvious. The invisible form (energy bars) was accepted without any problems by both men and women even though the respondents had been informed in advance about the flour of edible insects in the bars.

When evaluating the visible form (puff pastry bars sprinkled with mealworm), the initial hesitation was apparent and the male respondents overcame that faster. After the first tasting, the respondents showed no more hesitation to test another sample. This could stem from the above-mentioned suppression of insect flavor. Therefore, it is possible to conquer the initial fear to consume edible insects and to consider them as an interesting and acceptable novel constituent of the diet.

CONCLUSION

This study has confirmed a positive evaluation of selected food with the addition of edible insect employing a questionnaire survey of the general public in the Czech Republic. It has been proved that people welcome the opportunity to taste the samples of edible insects and do not exclude the possibility of conscious eating of the edible insect in the future. Regarding the conscious consumption of visible mealworm larvae on the puff pastry bars, the respondents associated their taste with known flavors of salty, sweet, and chicken, which may lead to further improvement in the attitude towards this commodity. Considering the examination of energy bars enriched with the cricket flour, both samples were positively evaluated. No statistically significant difference has been found between these two samples. Therefore, the results have shown that consumers in the Czech Republic are inclined to consume edible insect products, particularly if they can select the preferable form of the product and the included species.

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THE EFFECT OF STORAGE ON THE QUALITY PARAMETERS OF BABY FOOD

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ABSTRACT
The aim of this paper was focused on the quality changes of baby food stored at different temperature (4 °C, 20 °C, 40 °C and 60 °C) for 15 days. During storage, chemical and nutritional parameters analysis were carried out. Commercial fruits based baby food are the products usually made with fruits, sugar, and variable additives. As the foodstuffs intended for particular nutritional uses, baby foods for infants and young children conform to a set of strict guidelines e.g. nutritional quality, the addition of additives, labeling. However, being an important supplement to children's diet and for their progressive adaptation to ordinary food, the nutritional quality of commercial fruits baby food is very important. Samples of commercial fruits baby food from the market and pharmacies were analyzed by parameters: pH, total soluble solids, moisture, total acidity, vitamin C, proteins, sugars, and lipids. All samples of baby food are produced by foreign companies since currently, no Kosovo manufacturers are producing this range of products. The nutritional quality parameters are important to assess the quality of the product and how it can be safely stored. However, as a precaution, storage remarks in the product labels should always be followed.

Keywords: baby food; nutritional content; chemical composition; fruits

INTRODUCTION
Commercial fruits based baby food are the products usually made with fruits, sugar, water, and variable additives. Nutrient-rich baby foods are required with particularly high standards of quality and safety expressed through product specifications or attributes. As the foodstuffs intended for particular nutritional uses, baby foods for infants and young children conform to a set of strict guidelines e.g. nutritional quality, the addition of additives, labeling **(Čížková et al., 2009; Calabretti et al., 2017; Azemi et al., 2019)**. Commercial fruits baby food is suitable for use during the complementary feeding period. These foods are specifically formulated with appropriate nutritional quality to provide additional energy and nutrients to complement the family foods derived from the local diet by providing those nutrients which are either lacking or are present in insufficient quantities **(Codex Alimentarius, 1991)**. However, being an important supplement to children's diet and for their progressive adaptation to ordinary food, the nutritional quality of commercial fruits baby food is very important. Fruits-vegetable based foods are easy to digest and offer high nutrient density. The nutrition value of baby food deeply depends on the composition, what raw materials are used and what are the proportions of fruit or vegetable content **(Zein, ElSayed Bhnsawy and Arafa, 2019; Seidel et al., 2015)**. There is much evidence that the quality and composition of commercial baby food may contribute to the present and future health benefits of young children. Since infants between 6 months and 3 years of age are rather limited in their food choices, the commercial baby foods serve as an important source of energy, basic nutrients, fiber, vitamins, and minerals and establish their taste and eating patterns **(Čížková et al., 2009)**.

Infant fruits play an important role in the complementary feeding period. Infant fruits are defined as "processed fruit-based foods" that are divided into "simple fruits which are or have to be reconstituted with milk or other appropriate nutritious liquids''. Nutrition and dietary habits during infancy and early childhood play a role in shaping eating habits and health later in adolescence and into adulthood **(Walker and Goran, 2015; Prchalová et al., 2016)**. In many countries, infant fruits are among the first foods that are introduced at the beginning of the complementary feeding period. In recent decades, the reduction of time to be dedicated to the preparation of home meals has already led to the appearance of ready-to-eat food products on the market. The foods specifically manufactured for infants and young children have had an evolution. Prepared baby foods and formulas, intended for use of children aged between 4 months and 3 years, provide an appealing alternative for working mothers **(García et al., 2015; Garcia et al., 2014)**.

The aim of this paper was focused on the quality changes of baby food stored at different temperature (4 °C, 20 °C, 40 °C, and 60 °C) for 15 days.

The imbalance of calories and nutrients in some of the baby foods necessitates encouraging breast feeding at least

during the first 6 months. The high protein contents may damage the kidney and the quantity and quality of protein in baby foods should be adjusted to simulate human milk. The protein quality may be improved by improving the processing and storage conditions. To reduce the risk of dental caries, baby foods in which sucrose should be replaced by glucose or lactose may be selected **(Al-Othman, Khan and Al-Kanhal, 1997; Kohlboeck et al., 2012; Jackson, 2015)**. Nutrition education of mothers and health workers in kindergarten on the selection and preparation of the right type of baby foods and weaning practices will go a long way in improving the nutritional status of infants and children in the country.

Samples of commercial fruits based baby food from the market and pharmacies were analyzed by parameters: pH, total soluble solids, dry matter, total acidity, vitamin C, sugars (total sugars, reducing sugars), proteins, and lipids. All samples of baby food are produced by foreign companies since currently, no Kosovo manufacturers are producing this range of products. This is the first research of this type in Kosovo and it should give us a novel result. The nutritional quality parameters are important to assess the quality of the product and how it can be safely stored. However, as a precaution, storage remarks in the product labels should always be followed.

Scientific hypothesis

The scientific hypothesis of this study was focused on the quality changes of baby food stored at different temperature (4 °C, 20 °C, 40 °C, and 60 °C) for 15 days. During storage, chemical and nutritional parameters analysis were carried out. Quality parameters content was estimated at the beginning of the experiment and subsequently at 15 days interval.

MATERIAL AND METHODOLOGY

Sampling Preparation

Samples of commercial fruits based baby food with trade name: HiPP (Apple and Berry fruits), HiPP (Plum), HiPP (Apple and Banana), HiPP (Pear), FRUTEK (Banana), and HELLO (Carrot and Apple) were purchased from the market and pharmacies in Kosovo during the period June 2019. After samples collection, they were stored at different temperatures (4 °C, 20 °C, 40 °C, and 60 °C) for 15 days.

0.5 g of the product was diluted in 10 mL of acidified distilled water (1%) and extracted for 10 minutes. The solution was centrifuged at 3000 rpm for 10 minutes. The solid fraction, separated from the supernatant, was then subjected to a new extraction step. The liquid phases were collected and brought to a final volume of 10 mL. All analyses were performed in triplicate.

Nutritional analysis

Nutrient analysis was done for all collected samples. Determination of nutritional properties of commercial fruits baby food with trade name: HiPP (Apple and Berry fruits), HiPP (Plum), HiPP (Apple and Banana), HiPP (Pear), FRUTEK (Banana), and HELLO (Carrot and Apple) were performed according to the standard methods of the **AOAC (2005)** and **AOAC (2016)**. Total soluble solids content (TSS) measured using the Abbe refractometer calibrate against sucrose and expressed in °Brix. Titratable acidity (TA) was measured according to AOAC Method **(AOAC, 2005; AOAC, 2016)** and expressed as milligrams of citric acid. pH was measured using pH/mv meter, and dry matter (DM) was measured in triplicate by drying 5 g of the fresh fruits at 105 °C until constant weight (4 – 6 hours). The determination of lipids was done by Soxhlet extraction after digestion of the samples by hydrochloric acid hydrolysis, followed by extraction of the fats with petroleum ether. After the extraction, lipid content was determined by weighing **(AOAC, 2005)**. Protein was determined by the Bradford method with some modifications. Gelatin is commonly used to create the standard curve, and the absorption is measured at 545 nm in a spectrophotometer. Reducing sugar was determined using the method of Lane and Eynon and Fehling's solution as described by AOAC Methods **(AOAC, 2005; AOAC, 2016)**. Total sugars were determined by the phenol sulfuric acid method **(Nielsen, 2009)**. Glucose is commonly used to create the standard curve, and the absorption is measured at 490 nm. Vitamin C content was estimated using a spectrophotometric method with 2,4-dinitrophenyl hydrazine as an indicator **(AOAC, 2005; AOAC, 2016)**. Samples were homogenized with metaphosphoric acid (5% metaphosphoric acid in 10% acetic acid solution in water), filtered and treated with 85% sulphuric acid solution and 2,4-dinitrophenyl hydrazine, and then incubated at 60 °C for 60 min in a water bath. Absorbance was measured at 520 nm in a spectrophotometer (Genesys 10S UV-Visible) for the estimation of vitamin C in the fruits.

Statistical analysis

All data were expressed as the mean ± standard deviation of triplicate experiments. All statistical analyses performed using the MS Excel program and SPSS 22.0 statistics software Differences were tested for significance using the ANOVA procedure, with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Samples of commercial fruits based baby food with trade name: HiPP (Apple and Berry fruits), HiPP (Plum), HiPP (Apple and Banana), HiPP (Pear), FRUTEK (Banana), and HELLO (Carrot and Apple) were purchased from the market and pharmacies in Kosovo during the period June 2019. The effect of storage on quality changes of baby food is given in Table 1, Table 2, and Figure 1.

The pH values of commercial fruits-based baby food were decreased during storage (Table 1). The average pH value ranging from 3.55 ±0.1 to 2.85 ±0.1 sample A2 HiPP (Plum), 3.88 ±0.1 to 3.68 ±0.1 to sample A4 HiPP (Pear), 3.93 ±0.1 to 3.69 ±0.1 sample A6 HELLO (Carrot and Apple), 3.94 ±0.1 to 2.94 ±0.1 sample A1 HiPP (Apple and Berry fruits), 3.98 ±0.1 to 3.55 ±0.1 sample A3 HiPP (Apple and banana) and highest 4.31 ±0.1 to 4.15 ±0.1 sample A5 FRUTEK (Banana). Statistical analysis showed that storage interval and temperature treatments had a significant ($p < 0.05$) effect on the pH value of all samples.

Table 1 The chemical composition of commercial fruits based baby food.

Sample	Temperature [˚C]	Time of Storage [Days]	pH	W(DM)/%	TSS/°Brix	TA/%	Vitamin C mg.100g^{-1}
A_1 HiPP(Apple/ Berry fruits)	4 °C	15	3.94 ±0.1	1.34 ±0.1	7.0 ±0.1	0.61 ±0.2	30 ±0.2
	20 °C		3.92 ±0.1	1.34 ±0.1	7.0 ±0.1	0.60 ±0.2	30 ±0.2
	40 °C		3.54 ±0.1	1.29 ±0.1	7.6 ±0.1	0.52 ±0.2	25 ±0.2
	60 °C		2.94 ±0.1	1.08 ±0.1	8.2 ±0.1	0.41 ±0.2	20±0.2
A_2 HiPP (Plum)	4 °C	15	3.55 ±0.1	3.35 ±0.1	6.2 ±0.1	0.55 ±0.2	30 ±0.2
	20 °C		3.53 ±0.1	3.35 ±0.1	6.2 ±0.1	0.55 ±0.2	30 ±0.2
	40 °C		3.10 ±0.1	3.20 ±0.1	6.8 ±0.1	0.49 ±0.2	25 ±0.2
	60 °C		2.85 ±0.1	3.00 ±0.1	7.5 ±0.1	0.40 ±0.2	20 ±0.2
A_3 HiPP(Apple/ Banana)	4 °C	15	3.98 ±0.1	2.24 ±0.1	5.0 ±0.1	0.58 ±0.2	8.9 ±0.2
	20 °C		3.98 ±0.1	2.24 ±0.1	5.0 ±0.1	0.56 ±0.2	8.9 ±0.2
	40 °C		3.85 ±0.1	2.15 ±0.1	5.6 ±0.1	0.45 ±0.2	8.1 ±0.2
	60 °C		3.55 ±0.1	2.04 ±0.1	6.2.0 ±0.1	0.38 ±0.2	7.2 ±0.2
A_4 HiPP (Pear)	4 °C	15	3.88 ±0.1	2.43 ±0.1	4.75 ±0.1	0.48 ±0.2	10 ±0.2
	20 °C		3.88 ±0.1	2.43 ±0.1	4.75 ±0.1	0.46 ±0.2	10 ±0.2
	40 °C		3.79 ±0.1	2.33 ±0.1	5.30 ±0.1	0.39 ±0.2	5.4 ±0.2
	60 °C		3.68 ±0.1	2.23 ±0.1	5.90 ±0.1	0.28 ±0.2	1.6 ±0.2
A_5 FRUTEK(Banana)	4 °C	15	4.31 ±0.1	2.61 ±0.1	5.1 ±0.1	0.35 ±0.2	8.7 ±0.2
	20 °C		4.31 ±0.1	2.61 ±0.1	5.1 ±0.1	0.35 ±0.2	8.7 ±0.2
	40 °C		4.25 ±0.1	2.52 ±0.1	5.7 ±0.1	0.28 ±0.2	7.6 ±0.2
	60 °C		4.15 ±0.1	2.43 ±0.1	6.4 ±0.1	0.21 ±0.2	6.3 ±0.2
A_6 HELLO(Carrot/ Apple)	4 °C	15	3.93 ±0.1	2.13 ±0.1	6.1 ±0.1	0.30 ±0.2	10 ±0.2
	20 °C		3.93 ±0.1	2.13 ±0.1	6.1 ±0.1	0.30 ±0.2	10 ±0.2
	40 °C		3.81 ±0.1	2.05 ±0.1	6.8 ±0.1	0.23 ±0.2	5.4 ±0.2
	60 °C		3.69 ±0.1	1.94 ±0.1	7.7 ±0.1	0.15 ±0.2	4.5 ±0.2

Note: Data are expressed as average value ± standard deviation of three replicates.

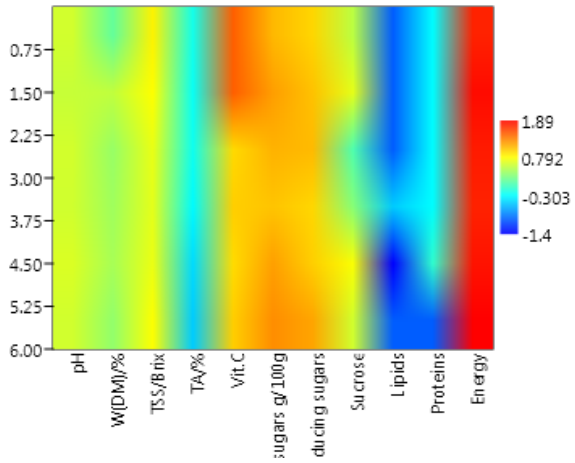

Figure 1 The 3D visualization data of chemical and nutritional parameters of baby food.

Our results correspond with the results of the work **Carbonell-Capella et al. (2014)**, **Zulueta et al. (2007)** reported that the pH values of commercial fruits-based baby foods in the range of 3.54 – 4.12 and pH range 2.96 – 4.11 respectively. Similar results reached **Usal and Sahan (2020)**, who report that the pH of the commercial baby food ranged from 3.55 – 5.24. **Touati et al. (2016)** reported that the pH values were significantly decreased during this period, independently of the temperature used. Prior storage, pH values were 3.88, 3.57, and 2.77. The pH of fruits is lower than vegetables and therefore vitamin C is less degraded in fruits **(Mesías-García, Guerra-Hernández, and García-Villanova, 2010)**.

The values for commercial fruits-based baby food of the total dry matter were decreased during storage (Table 1). The average value ranged from 1.34 ±0.1 to 1.08 ±0.1 sample A1 HiPP (Apple and Berry fruits) and increasing on other samples: 2.13 ±0.1 to 1.94 ±0.1 sample A6 HELLO (Carrot and Apple), 2.24 ±0.1 to 2.04 ±0.1 sample A3 HiPP (Apple and banana), 2.43 ±0.1 to 2.23 ±0.1 sample A4 HiPP (Pear), 2.61 ±0.1 to 2.43 ±0.1 sample A5 FRUTEK (Banana) and to be higher value is 3.35 ±0.1 to 3.35 ±0.1 sample A2 HiPP (Plum). Statistical analysis showed that storage interval and temperature treatments had a significant (p <0.05) effect on the total dry matter value of all samples.

Usal and Sahan (2020), reported that the dry matter levels of samples were varied between 81.41 and 93.10% depending on ingredients. When the dry matter of the samples was examined, the highest value was found in 14 (93.10%) sample and the lowest value in 11 (81.41%). These values are much higher than our results.

An increase was observed in soluble solids TSS/°Brix of samples of commercial fruits baby food throughout the storage (Table 1). The average value ranged from 4.75 ±0.1 to 5.90 ±0.1 sample A4 HiPP (Pear), and increasing on other samples: 5.0 ±0.1 to 6.2 ±0.1 sample A3 HiPP (Apple and banana), 5.1 ±0.1 to 6.4 ±0.1 sample A5 FRUTEK (Banana), 6.1 ±0.1 to 7.7 ±0.1 sample A6 HELLO (Carrot and apple), 6.2 ±0.1 to 7.5 ±0.1 sample A2 HiPP (Plum) and highest 7.0 ±0.1 to 8.2 ±0.1 sample A1 HiPP (Apple and Berry fruits). The increase in soluble solids TSS/°Brix value may be due to an increase in temperature and inversion of sucrose into glucose and fructose. Statistically, storage interval and treatments had significantly (p <0.05) effect on soluble solids TSS/°Brix value of all samples during storage.

Different authors have observed that the TSS/°Brix value increased during storage at different temperatures. **Zulueta et al. (2007)** reported that the TSS/°Brix ranged from 14.0 to 24.9. **Touati et al. (2016)** reported a significant increase in TSS/°Brix was observed for all the samples during storage 11.60 – 15.20. **Carbonell-Capella et al. (2014)** reported that the TSS/°Brix ranged from 12.0 to 24.9. Our results were the lowest of these studies.

The total acidity in samples of commercial fruits baby food was decreased during the storage period (Table 1). The average value for total acidity is lower ranged from expressed in % of citric acid and ranged from 0.30 ±0.2 to 0.15 ±0.2 sample A6 HELLO (Carrot and Apple) while the highest values of total acidity are in the sample A1 HiPP (Apple and Berry fruits) 0.61 ±0.2 to 0.41 ±0.2. The reason behind the increase in acidity is the degradation of non-reducing sugar which increases the acidity. Total acidity was statistically significantly (p <0.05) affected by storage intervals and temperature treatments.

Organic acids in fruits exhibit a low susceptibility to changes during processing and storage, combined with adequate stability compared to pigments and flavor compounds **(Fügel, Carle and Schieber, 2005)**. Many authors have been observed that fruit-based products showed acidic properties in studies conducted. The total acidity showed a decrease during storage in their studies **(Zulueta et al., 2007; Jie et al., 2013; Batkan, Kundakçi, and Ergönül, 2012)**. Our findings are similar to those found by them. Our results correspond with the results of the studies **Usal and Sahan (2020)** reported that the total titratable acidity (g of citric acid mg.100g^{-1}) was between 0.15 and 0.54 mg.100g^{-1}. Similar results observed **Carbonell-Capella et al. (2014)**, who report that the total acidity (g of citric acid per 100 g) of the commercial baby food was between 0.308 and 0.533 g of citric acid per 100 g.

Table 2 The nutritional composition of commercial fruits based baby food.

Sample	Temperature [°C]	Time of Storage [Days]	Total sugars g.100g^{-1}	Reducing sugars g.100g^{-1}	Lipids g.100g^{-1}	Proteins g.100 g^{-1}
A_1 HiPP(Apple/ Berry fruits)	4 °C	15	12.4 ±0.1	9.2 ±0.1	0.1 ±0.1	0.5 ±0.1
	20 °C		12.4 ±0.1	9.2 ±0.1	0.1 ±0.1	0.5 ±0.1
	40 °C		11.4 ±0.1	10.2 ±0.1	0.1 ±0.1	0.5 ±0.1
	60 °C		10.2 ±0.1	11.3 ±0.1	0.1 ±0.1	0.5 ±0.1
A_2 HiPP (Plum)	4 °C	15	16.0 ±0.1	11.2 ±0.1	0.1 ±0.1	0.5 ±0.1
	20 °C		16.0 ±0.1	11.2 ±0.1	0.1 ±0.1	0.5 ±0.1
	40 °C		15.1 ±0.1	12.3 ±0.1	0.1 ±0.1	0.5 ±0.1
	60 °C		13.9 ±0.1	13.4 ±0.1	0.1 ±0.1	0.5 ±0.1
A_3 HiPP(Apple/ Banana)	4 °C	15	13.2 ±0.1	12.1 ±0.1	0.1 ±0.1	0.5 ±0.1
	20 °C		13.2 ±0.1	12.1 ±0.1	0.1 ±0.1	0.5 ±0.1
	40 °C		12.2 ±0.1	13.2 ±0.1	0.1 ±0.1	0.5 ±0.1
	60 °C		11.1 ±0.1	14.3 ±0.1	0.1 ±0.1	0.5 ±0.1
A_4 HiPP (Pear)	4 °C	15	11.0 ±0.1	9.10 ±0.1	0.3 ±0.01	0.5 ±0.1
	20 °C		11.0 ±0.1	9.10 ±0.1	0.3 ±0.01	0.5 ±0.1
	40 °C		10.1 ±0.1	10.2 ±0.1	0.3 ±0.01	0.5 ±0.1
	60 °C		9.0 ±0.1	11.3 ±0.1	0.3 ±0.01	0.5 ±0.1
A_5 FRUTEK(Banana)	4 °C	15	15.8 ±0.1	9.9 ±0.2	0.04 ±0.1	0.8 ±0.1
	20 °C		15.8 ±0.1	9.9 ±0.2	0.04 ±0.1	0.8 ±0.1
	40 °C		14.9 ±0.1	10.8 ±0.2	0.04 ±0.1	0.8 ±0.1
	60 °C		13.1 ±0.1	11.9 ±0.2	0.04 ±0.1	0.8 ±0.1
A_6 HELLO(Carrot/ Apple)	4 °C	15	18.9 ±0.1	15.0 ±0.1	0.1 ±0.1	0.1 ±0.1
	20 °C		18.9 ±0.1	15.0 ±0.1	0.1 ±0.1	0.1 ±0.1
	40°C		17.5 ±0.1	16.1 ±0.1	0.1 ±0.1	0.1 ±0.1
	60 °C		16.3 ±0.1	17.2 ±0.1	0.1 ±0.1	0.1 ±0.1

Note: Data are expressed as average value ± standard deviation of three replicates.

Samples of commercial fruits baby food are a good source of Vitamin C. Some sample is fortified with Vitamin C and contains high value of Vitamin C. The stability of Vitamin C decreases with increasing temperature. A decrease in Vitamin C in samples of commercial fruits baby food during storage (Table 1). The highest average value of Vitamin C is A1 HiPP (Apple and Berry fruits) 30 ±0.2 to 20 ±0.2 also and sample A2 HiPP (Plum) 30 ±0.2 to 20 ±0.2. The relatively high amounts of Vitamin C also contain the samples: A4 HiPP (Pear) 10 ±0.2 to 1.6 ±0.2 and sample A6 HELLO (Carrot and Apple) 10 ±0.2 to 4.5 ±0.2, while the samples A3 HiPP (Apple and banana) value of Vitamin C is lower 8.9 ±0.2 to 7.2 ±0.2 and lower in the sample A5 FRUTEK (Banana) 8.7 ±0.2 to 6.3 ±0.2. Statistically, storage interval and treatments had significantly (p <0.05) effect on the Vitamin C value of all samples during storage.

It is known that losses of vitamin C occur during processing and storage. That these losses may be used as an indicator of the aggression to nutritional value suffered in the industrial or culinary process **(Mesías-García, Guerra-Hernández and García-Villanova, 2010)**. It is thought that vitamin C undergoes oxidation and substantial losses during processing, storage, and heat treatment of fruit and vegetables during the processing periods **(El-Ishaq and Obirinakem, 2015)**. To prevent such losses, vitamin C may be added to vegetable and fruit-based baby foods, and optimization of the pH value. If the pH value increases from 3 to 6, vitamin C oxidation increases **(Fan, 2005)**. **Uğur et al. (2020)** reported that the determined concentrations ranged from 0.1 to 8.5 mg.100g^{-1} in samples that were not-fortified with vitamin C. Also, the concentration of vitamin C was very low in samples that were not containing added vitamin C compared to other fruit and vegetable-based baby foodstuffs. **Silva et al. (2018)** found that vitamin C concentration in baby foods ranged from 1.50 to 144 mg.100g^{-1} and the measured amount of vitamin C was 50% higher than the reported concentration on the label. In the same study, all values were within the allowed limits in compliance with EU legislation. **Brandon et al. (2014)** revealed that determining vitamin C concentrations ranged from 97 to 147% of the declared concentration in infant formula. In the study conducted by **Mesías-García, Guerra-Hernández and García-Villanova (2010)** for vegetable-based baby foods, no vitamin C was found in any of the products and it was thought that naturally found vitamin C was completely lost during processing. **Carbonell-Capella et al. (2014)** revealed that vitamin C contents were between 1.9 and 71.5 mg.100g^{-1} in 23 fruit-based baby foods and the lowest vitamin C amounts (0 – 2.4 mg.100g^{-1}) were determined in fruit-based baby foodstuffs which were not-fortified with vitamin C. When the declared amount was subtracted from the analyzed amount, very small amounts of vitamin C were found in the samples. It is thought that vitamin C undergoes substantial losses in the processing periods. Our findings are congruent with the measured amount of vitamin C was generally the reported amount in vitamin C added and declared commercial fruits baby food. Our findings are congruent with the above-mentioned studies that the measured amount of vitamin C decreased during storage time and heat treatment.

Also, commercial fruits based baby food as a good source of sugars, and consequently, they are a good source of energy. A decrease was observed in the total number of sugars on these samples during storage (Table 2). The average value varied from 11.1 ±0.1 to 9.0 ±0.1 sample A4 HiPP (Pear), 12.4 ±0.1 to 10.2 ±0.1 sample A1 HiPP (Apple and Berry fruits), 13.2 ±0.1 to 11.1 ±0.1 sample A3 HiPP (apple and banana), 15.8 ±0.1 to 13.1 ±0.1 sample A5 FRUTEK (Banana), 16.0 ±0.1 to 13.9 ±0.1 sample A2 HiPP (plum) as well as higher in the sample A6 HELLO (carrot and apple) 18.9 ±0.1 to 18.3 ±0.1. During storage in different temperatures, sucrose in fruits is continuously converted into fructose and glucose which results in a reduction in the total number of sugars. Results demonstrated that storage and temperature treatment have a significant (p <0.05) effect on samples of baby food.

An increase was observed in reducing sugars during storage and temperature treatment (Table 2). The average value varied from 9.1 ±0.1 to 11.3 ±0.1 sample A4 HiPP (Pear), 9.2 ±0.1 to 11.3 ±0.1 sample A1 HiPP (Apple and Berry fruits), 12.1 ±0.1 to 14.3 ±0.1 sample A3 HiPP (Apple and banana), 9.9 ±0.1 to 11.9 ±0.1 sample A5 FRUTEK (Banana), 11.2 ±0.1 to 13.4 ±0.1 sample A2 HiPP (Plum) as well as higher in the sample A6 HELLO (Carrot and apple) 15.0 ±0.1 to 17.2 ±0.1. A raise in reducing sugars is due to the inversion of sucrose to reducing sugar because of acids. A conversation of pectin into fructose and glucose because of the rise in temperature during storage was observed. Storage and temperature treatment results were significant (p <0.05).

Many products that are frequently marketed to and consumed by infants and young children contain sugars that are far more than what is considered nutritionally beneficial and/or different from that stated on the nutrition label **(Walker and Goran, 2015)**. Total sugar content is positively correlated with fruit and vegetable content in commercial baby foods, particularly in spoonable foods (6.8 g sugar per 100 g), suggesting that they may be used as sweetening agents **(Garcia, McLean and Wright, 2016)**.

Our results were in concordance to those found by **Batkan, Kundakçi and Ergönül (2012)** reported that the average of total sugars was 9.94 – 10.97 g.100g^{-1} and that the average of reducing sugars was 8.39 – 10.08 g.100g^{-1}. It was found that the storage period significantly affected the total sugar and reducing sugar contents of all samples (p <0.05). In the period of storage, total sugar and reducing sugar contents of all samples showed an increasing trend. It is thought that this increase is related to the degradation of starch to glucose and maltose.

The values of proteins to samples of commercial fruits based baby food are lower. Fruits baby food do not count as protein foods and varied between 0.5 ±0.1 to sample A6 HiPP (Pear), 0.5 ±0.1 to sample A3 HiPP (Apple and Berry fruits), 0.5 ±0.1 to sample A5 HiPP (Apple and banana), 0.5 ±0.1 g.100g^{-1} to sample A4 HiPP (Plum) and the lowest of samples A8 HELLO (Carrot and apple) 0.1 ±0.1 while the highest 0.8 ±0.1 to sample A7 FRUTEK (Banana). No change in protein values was observed during storage and temperature treatment and statistically is significantly (p <0.05).

Also, commercial fruits based on baby food are not fat-rich foods. The content of lipids is low and varies from 0.04 ±0.01 to sample A7 FRUTEK (Banana), while in other samples its value is 0.1 ±0.01 to samples A3 HiPP (Apple and Berry fruits), A4 HiPP (Plum), A5 HiPP (Apple and banana), A8 HELLO (Carrot and apple)), while the highest value is in the sample A6 HiPP (Pear) 0.3 ±0.01. Also, no change in lipids values was observed during storage and temperature treatment and statistically is significantly (p <0.05).

It is very difficult to compare data with other authors because there is limited data in the literature about proteins and lipids to samples of commercial fruits-based baby food. Our results were the first in this study.

The obtained results can be useful in clarifying the changes in the quality of commercial fruits based baby food during storage in different temperature treatments (4 °C, 20 °C, 40 °C, 60 °C).

CONCLUSION

The samples of commercial baby food based fruits stored at refrigeration condition (4 °C) and room temperature (20 °C) had the maximum quality and maximum nutrients stability as compared to the treatment of increased temperature (40 °C and 60 °C) during 15 days of storage.

Samples of commercial fruits based baby food with trade name: HiPP (Apple and Berry fruits), HiPP (Plum), HiPP (Apple and Banana), HiPP (Pear), FRUTEK (Banana), and HELLO (Carrot and Apple) were purchased from the market and pharmacies in Kosovo during the period June 2019. However, as a precaution, storage remarks in the product labels should always be followed. All samples of baby food are produced by foreign companies since currently, no Kosovo manufacturers are producing this range of products.

This is the first research of this type in Kosovo and it should give us a novel result. Nutrition education of mothers and health workers in kindergarten on the selection and preparation of the right type of baby foods and weaning practices will go a long way in improving the nutritional status of infants and children in the country.

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MATHEMATICAL MODELING OF THE OIL EXTRUSION PROCESS WITH PRE-GRINDING OF RAW MATERIALS IN A TWIN-SCREW EXTRUDER

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ABSTRACT
The extrusion process of oil-containing raw materials using a twin-screw extruder is becoming increasingly common in food technology. The problem of high energy costs for the implementation of this process is solved by reducing the resistance of the process mass due to the preliminary grinding of raw materials. The classical theory of extrusion is based mainly on the use of theoretical solutions of mathematical models of processes, which are simplified and allow determining integral parameters using coefficients, the preparation of which for the calculation of the corresponding processes and equipment is a rather complicated and approximate procedure. Mathematical modelling of the movement of the technological medium at the individual stages of the processing of raw materials allows us to determine the analytical dependences for the power and energy parameters of the system and to carry out their effective technical and economic evaluation. Using the methods of mathematical analysis and data processing in the MathCAD software environment, graphical dependences of the power and energy parameters of the research technical system were obtained. By increasing the density of the oil-containing raw materials, which is extruded in the research extruder by 40.5% the pressure force increases by 41%, that is, there is an almost proportional relationship between the pressure force and the density of the processed raw material. With an increase in the angular velocity of the drive shaft ω more than 8 rad.s^{-1}, the pressure force in the research process increases sharply. With an increase in the density of raw materials, it is grinded before extrusion by 40%, the power consumption for the grinding process increases by 2.8 times for the recommended operating mode. Energy losses for pressing completely grinded raw materials are reduced by 2.52 times.

Keywords: process; extrusion; oil-containing; raw materials; energy consumption; extruder

INTRODUCTION
The theory of extrusion was developed mainly for the case of processing synthetic materials, which throughout the volume have a relatively uniform molecular weight and are characterized by a high level of dissipation of mechanical energy **(Shi et al., 2019)**. In the overwhelming majority of works on modelling the single-screw extrusion process, the inverse screw model is considered, which allows us to reduce the problem to a rectangular coordinate system, considering the screw to be stationary and unfolded on the plane, and the working cylinder to be moving relative to the screw and also unfolded on the plane **(Martin and Otakar, 2017)**. The classical theory of extrusion is based on the use of theoretical solutions of mathematical models of processes, which are simplified and allow us to determine, basically, integral parameters. In a significant part of the work, coefficients are introduced, the obtaining of which is rather difficult to calculate the corresponding processes and equipment **(Kudrin, 1997)**. The use of computer technology allows us to analyse more complex models, which make it possible to assess the influence of several factors on the extrusion process, but in many cases, it does not lead to significant refinement of the results, it requires the selection of factors that most significantly affect the extrusion process and satisfy the requirements of designers **(Herman, 1975)**. Many researches are devoted to the research of viscous-plastic mass flows along a conical channel, in cylindrical pipes, in channels of a rectangular, square, elliptical, screw, and complex section. In some works, the movement of viscoplastic food materials was analysed based on ideas about the dosing zone, and the effect of wall sliding was estimated **(Petrov and Slavnov, 2013)**. The research twin-screw press extruders are distinguished by longer helical shafts, due to the introduction of special mixing and grinding elements in their designs. In scientific works **(Palamarchuk et al., 2020; Zeleňáková et al., 2019; Shahbaz et al., 2017)**, based on researches conducted under industrial conditions, a dosing zone is described based on hydrodynamic analysis of flows within individual C-shaped volumes **(Sheiko et al., 2019)**. The leakage flows through gaps, compression of raw materials, the distribution of pressure in the channel and the influence of these factors on the

performance of the extruder as a whole are considered. In many scientific works **(Mushtruk et al., 2020; Tsagareishvili et al., 2019; Zheplinska et al., 2019)** the rheology of the behavior of raw materials is described in sufficient detail, formulas are given for the design calculation of twin-screw extruders, and the effect of mixing and crushing working bodies of twin-screw extruders on the processing of rubber compounds and plastics is described. Effective mathematical modelling of twin-screw extruders has long been limited to the development of geometric parameters and the justification of processing modes, based on practical experience and experimental data, due to the rather complicated design of the executive bodies **(Pugachev, Levina and Shalaeva, 2011)**.

The aim of this scientific work is a feasibility study of the implementation of the stage of preliminary grinding of oil-containing raw materials in the extrusion process of oil by a twin-screw extruder based on a theoretical graph-analytical analysis of the power and energy parameters of the process. To achieve this aim it is necessary to solve the following tasks:

- determination and assessment of power and energy characteristics for the implementation of the mechanical extrusion of the liquid fraction of oil-containing raw materials;
- obtaining graphical interdependencies of the research factors for the developed extruder circuit;
- substantiation of regime power and kinematic parameters of the executive bodies of the developed extruder.

Thus, the theoretical justification of the power and energy parameters during the implementation of individual stages of the extrusion process of oil-containing raw materials to reduce energy consumption and increase oil yield is quite an urgent task, which is solved in this scientific research.

Scientific hypothesis

Grinding of oil-containing raw materials before pressing and selection of the optimal operating mode of the extruder can increase the efficiency of its operation – increase the pressing force and oil yield and reduce energy consumption for the process.

MATERIAL AND METHODOLOGY

When operating screw presses, from the beginning to the end of the pressing of seeds, it passes from one physical state to another until the oil and oil meal come out. To research these processes, they used the method of separation or decomposition, examining them in stages. Although the individual stages are interconnected, each of them in a complex system of processing of raw materials can occur simultaneously, parallel or sequentially, which happens inside the extruder's press path, where the operations of transportation, grinding, mixing, heating and extrusion of the raw material are carried out.

When conducting a theoretical analysis and justification of the power, torque and energy characteristics of the developed twin-screw apparatus for oil extrusion, methods of mathematical analysis and data processing in the MathCAD software environment were used to obtain the necessary graphical and analytical dependencies describing the main operating parameters of the system.

Statistical analysis

The statistical evaluation of the results was carried out by standard methods using statistical software Statgraphics Centurion XVII (StatPoint, USA) – multifactor analysis of variance (MANOVA), LSD test. Statistical processing was performed in Microsoft Excel 2016 in combination with XLSTAT. Values were estimated using mean and standard deviations.

RESULTS AND DISCUSSION

In scientific works **(Kehinde, 2016; Sokolov et al., 2018)** various schemes of the process of reception of oils from seeds of various oil-containing cultures which have several lacks are presented **(Palamarchuk et al., 2019; Prashanth et al., 2019)**. The general scheme of the process of obtaining oil from seeds of oil-containing crops in a twin-screw press extruder (Figure 1) can be described as follows. A raw material (seeds of oil-containing cultures) through the neck of the hopper *1* by gravity are sent to the loading chamber of the extruder and fills the free space between the inner wall of the chamber and screw turns. The seeds are captured by the coils of the screw nozzles *2* and are moved to the heating chamber *3*, where it is gradually compressed, and in the area of the cam nozzles *4* is partially grinded and heated from the walls of the chamber. Then the raw material enters the grain chamber *5*, where it is pressed. Part of the oil flows through the grain of the hole, and the remaining raw materials are transported to the next extraction zone – heating chambers and extrusion. Screw nozzles along with the shaft alternate with grinding. To increase the yield of oil, pressing occurs in several stages with their repetition in the extrusion path. In the area of the cone nozzles *6* and matrix *7*, the oil meal is removed from the extruder in the form of petals or granules.

When describing the screw pressing mechanism, the principle of dividing it into sections is applied. In scientific works **(Polosin and Chistyakova, 2014; Shirazian et al., 2017; Chen et al., 2019)** this principle of division into sections was not used. A section can end with a matrix or a compression shutter – a section of the mechanism on which the screw turn is interrupted and the passage section of the extrusion path of the extruder is reduced **(Owolarafe, Osunleke and Odejobi, 2008)**.

In accordance with the specified method of separation into sections or elementary screw mechanisms with conditionally constant parameters of the pressing process, the press path of the research twin-screw press extruder was divided into four conditional sections: I, II, III, and IV. Each section ends with a section of the mechanism on which the bore of the extrusion path of the press is reduced or increased within the same type of working bodies. In section *I* there is a displacement of the material in the auger channel, in section *II* there is a partial grinding of production in the area of the triangular cam nozzles and the movement of the raw material in the compression gate, in section *III* – the extrusion of oil through the holes in the grain chambers, and ie section *IV* – the displacement of the material in the matrix.

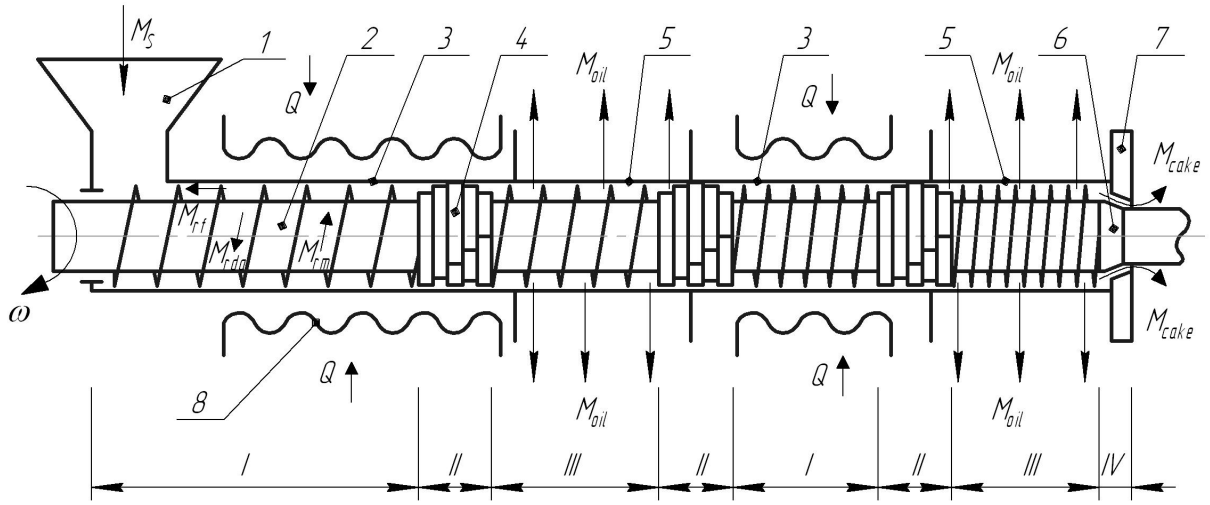


Figure 1 The design scheme of a twin-screw press extruder. Note: 1 – hopper, 2 – twin screw, 3 – heating chamber, 4 – grinding elements (nozzles), 5 – grain chamber, 6 – cone nozzle, 7 – matrix, 8 – electrical heating. Note: M_S – a mass of seeds; Q – the amount of heat supplied to the raw material; M_{rm} – a mass of the main flow of raw materials; M_{oil} – a mass of the pressed oil; M_c – a mass of oil cake; M_{rf} *a* – mass of the return flow of raw material directed along the gap between the cylinder and the screw; M_{rda} a – mass of the return flow of raw material directed along the channel of the profile of the auger.

Assuming that in the research process grinding of seeds is realized in the radial direction to the center of the seeds, it is possible to determine the geometric parameters of the grinding unit of the extruder, which are presented in Figure 2.

Using the calculated scheme of the interaction of the cams (Figure 3) we obtained that, $\sin\frac{\alpha}{2} = 0{,}5\sqrt{\frac{d_s}{r_{ge}}}$

In the research system, three main forces can be noted: grinding force F_P; a force of pressure created in the screws of the screw F_S; friction force F_f. The direction of the grinding force is taken normal to the seed, which is crushed (Figure 2). In scientific works **(Bako, Enyi, and Imolemhe, 2020; Soares, Zhang and Sacks, 2017)** the force of pressure created in the screws of the auger and the force of friction was determined, and in scientific works **(Bogaert et al., 2018; Tuta and Palazoğlu, 2017; Herak, 2013)** the force of friction and the force of demand were determined.

Given the regularities of the mechanics of movement of the executive bodies and the geometric characteristics of the extruder presented above (Figure 2), we write the expression for the projection of the pressure force on the *ox* axis in the form:

$$F_x = 0.393 n_S (D_S^2 - d_S^2) P_{\max} = 3.75 \omega (D_S^2 - d_S^2) P_{\max} \qquad (1)$$

Where: n_S and ω – respectively, the speed and angular velocity of the screw shaft; D_S – the outer diameter of the screw auger*;* d_S – the outer diameter of the auger body; P_{max} – the maximum pressure that develops the auger.

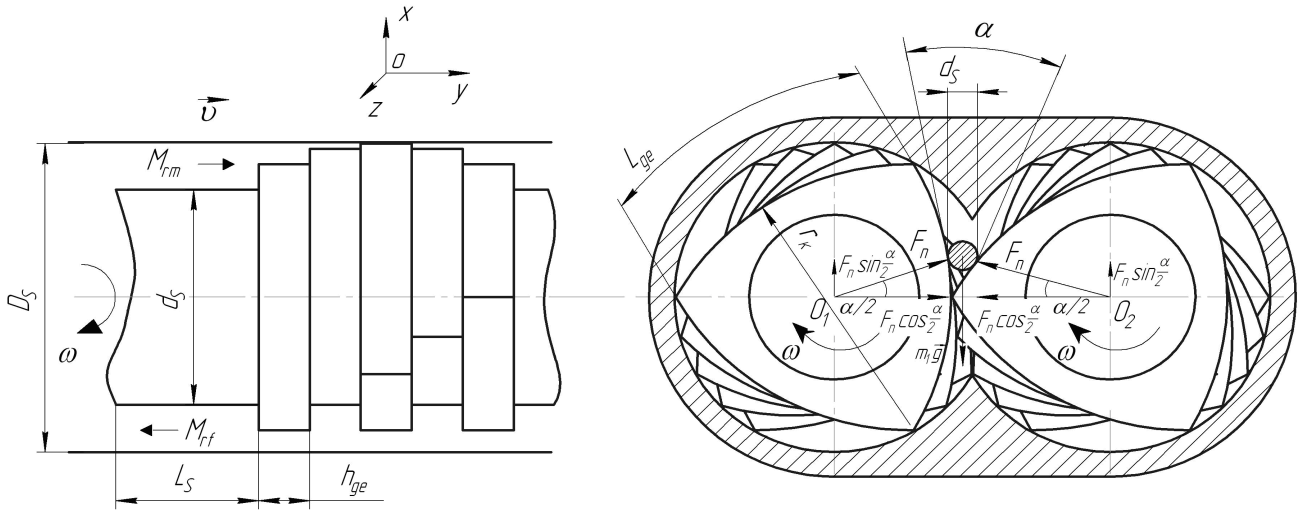


Figure 2 Scheme for determining the geometrical parameters of the seed grinding unit in triangular cam nozzles. Note: D_S – the outer diameter of the screw coil, d_S – the outer diameter of the screw body, L_S – the length of the screw nozzle, L_{ge} – the width of the cam nozzle, L_{ge} – the width of the cam nozzle length of cam nozzle, O_1, O_2 – centers of cams; r_{ge} – the radius of the cam; d_s – diameter of the seed, ω – the angular velocity of the shaft.

The friction force of raw materials on the inner surface of the extruder body can be described by the formula:

$$F_f = k \cdot F_d \cdot F_{ad} \tag{2}$$

Where: k_f – the coefficient of friction of the raw material on the inner surface of the housing; F_d – a force of deformation of raw materials: $F_d = F_x$; F_{ad} – adhesive force, which takes into account the adhesion of the product with the parts of the press is determined by the adhesive experimentally.

The projections of the momentum of the elements of the system under study along the coordinate axes are:

$$m_1 \upsilon_z = \left(F_S - F_S \cdot k_t - F_{ad}\right) t_1 \tag{3}$$

Where: m_1 – the mass of raw material;

$$m_1 \upsilon_x = \left(2F_P \sin\frac{\alpha}{2} + F_x - F_x \cdot k_t - m_1 g - F_{ad}\right) t_2 \tag{4}$$

It is obvious that $m_1 \upsilon_y = 0$

The axial velocity of the raw material along the auger channel can be determined by the formula

$$m_1 \upsilon_x = \left(2F_P \sin\frac{\alpha}{2} + F_x - F_x \cdot k_t - m_1 g - F_{ad}\right) t_2 \tag{5}$$

$$\upsilon_z = \frac{z \cdot n_S}{60} = \frac{z \cdot \omega}{2\pi}$$

The time of promotion of products to the section with grinding cams is $t_1 = \frac{z}{\upsilon_z}$, and at $z_{\max} = L_S$ that time will increase to $t_1 = \frac{L_S}{\upsilon_z}$. The grinding time of products between the surfaces of the cams is $t_2 = \frac{x}{\upsilon_x}$, and at $x_{\max} = L_{ge}$ this time is calculated as follows $t_2 = \frac{L_{ge}}{\upsilon_x}$.

Using the presented equations, the projections of the mass forces of a mechanical system on the coordinate axis can be represented as:

$$F_z = \frac{(F_S(1-k_t) - F_{ad})}{m_1} = \frac{\upsilon_z}{t_1} = \frac{\upsilon_z^2}{z} \tag{6}$$

Where: z – the current coordinate.

$$F_x = \frac{(2F_P \sin\frac{\alpha}{2} + F_x(1-k_t) - m_1 g - F_{ad})}{m_1} = \frac{\upsilon_x}{t_2} = \frac{\upsilon_x^2}{x} \tag{7}$$

Where: x – the current coordinate; g – the acceleration of gravity.

Obviously a component $F_y = 0$

Based on the developed calculation scheme of the grinding unit, efforts were found to grind the raw materials from the equation of conservation of the amount of its movement.

$$m_1 \upsilon_x = \left(2F_P \sin\frac{\alpha}{2} + \frac{P_x}{S}(1-k_r) - m_1 g - F_{ad}\right)\frac{L_{ge}}{\upsilon_x} \tag{8}$$

Then the grinding force can be found by the equation:

$$F_P = \frac{1}{2\sin\frac{\alpha}{2}}\left[\frac{m_1 \omega^2 r_{ge}^{\ 2}}{L_{ge}} - \frac{\omega^2 r_{ge}^{\ 2} \rho \ln L_K}{L_{ge} z_{ge} h_{ge}}(1-k_t) + m_1 g + F_{ad}\right] \tag{9}$$

or

$$F_P = \frac{1}{2\sin\frac{\alpha}{2}}\left[\frac{\omega^2 r_{ge}^{\ 2}}{L_{ge}}\left(m_1 - \frac{\rho \ln L_{ge}}{z_{ge} h_{ge}}\right)(1-k_t) + m_1 g + F_{ad}\right] \tag{10}$$

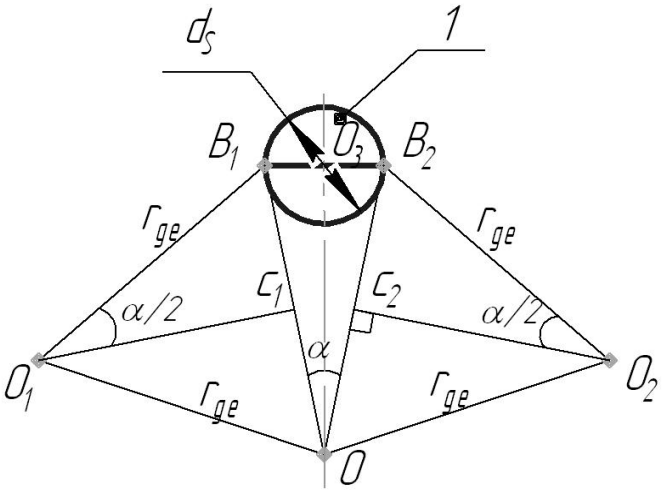


Figure 3 Settlement scheme of interaction of grinding cams. Note: *1* – seed; O_1, O_2 – the centers of the cams (grinding elements); r_{ge} – the radius of the cam (grinding elements); O_3 – the center of the seed; d_s – the diameter of the seed.

Based on the obtained equations and using the MathCAD software environment, graphical dependences for the force and power characteristics of the research process were obtained (Figure 4), of which it is evident that with increasing the density of the extruded slurry by 40.5%, the pressure force increases by 41%, i.e. there is a proportional relationship between the pressure force and the density of the raw material processed. In work **(Anderegg et al., 2019)** was installed the pressure force increases by 31%, in the work **(Troshin, 2017)** was installed the pressure force increases by 34.2%, in the work **(Leray, et al., 2016)** was installed the pressure force increases by 24.2%. With an increase in the angular velocity of the drive shaft ω more than 8 rad.s^{-1}, the pressure force on the raw material increases sharply, which justifies the recommended angular speed of rotation of the extruder shaft in the range ω = 4 – 7 rad.s^{-1}. The authors of the following scientific works **(Yang et al., 2018; Zhou et al., 2019)** recommend the angular velocity of rotation of the extruder shaft in the range ω = 8 – 12 rad.s^{-1}, but rational is the speed not exceeding 7 rad.s^{-1}. For the recommended regime of rotational movement of the extruder's executive bodies, the force of grinding products by working rolls F_p increases 3 times (Figure 4, a) during the extraction of raw materials, the density of which increases by 40%. Moreover, this force varies within 1.5 – 6 kH. The authors of the following scientific works **(Syryamkin et al., 2019; Kolyanovska et al., 2019)** investigated that the grinding force ranges from 5.5 to 16 kN.

With an increase in the density of raw materials, it is grinded before being extruded by 40%, the power consumption for the grinding process increases by 2.8 times and amounts to 0.7 – 2 kW for the recommended operating mode (Figure 4, b). In scientific works **(Cai and Liu, 2017; Mamanpush et al., 2018)** it is proved that with an increase in the density of raw materials, it is grinded before being extruded by 30%, the power consumption for the grinding process increases by 0.8 – 1.2 times and amounts to 0.1 – 0.8 kW **(Cherednichenko and Bal-Prylypko, 2019; Zheplinska et al, 2020)**.

To evaluate the effectiveness of preliminary grinding of seeds in the process of oil extrusion, the following calculation procedure was used **(Iuga et al., 2016)**, according to which the pressure is determined by the formula:

$$P = c \cdot \rho^m, Pa \quad (11)$$

For whole seeds $c = 1.76 \cdot 10^{-3}$, $m = 6.66$, that is $P_0 = 1.76 \cdot 10^{-3} \cdot \rho_0^{6.66}$, and for grinded seeds $c = 3.3 \times 10^{-32}$, m = 11.84, that is $P_1 = 3.3 \cdot 10^{-32} \cdot \rho_1^{11,84}$

Where: ρ_0 – density of whole seeds, ρ_0 = 1000 – 1050 kg.m^{-3}; ρ_1 – density of grinded seeds, ρ_1 = 1110 – 1150 kg.m^{-3}.

The ratio of the power consumption for pressing whole seeds N_0 and grinded N_1 is:

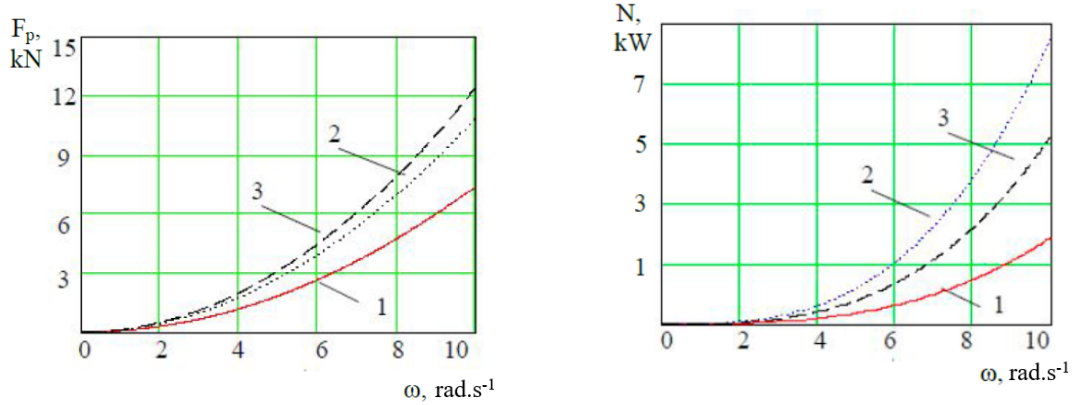
$$k_N = \frac{N_0}{N_1} = \frac{P_0}{P_1} = \frac{1.76 \cdot 10^{-13} \cdot \rho_0^{6.66}}{3.3 \cdot 10^{-32} \cdot \rho_1^{11.84}} = 294 \quad (12)$$

When the energy efficiency coefficient k_N was introduced to evaluate the research process, it was found that the coefficient c is inaccurate for the grinded material. After clarification for the grinded raw materials $c = 3.86 \times 10^{-30}$.

$$\text{Then} \quad k_N = \frac{N_0}{N_1} = \frac{P_0}{P_1} = 2.52$$

That is, the energy loss for pressing the grinded material for the research process of oil extrusion is reduced by 2.52 times. The rational design and technological parameters of the modernized press extruder were determined based on the experimental researches, which were: the temperature of the first and second heating zones of the buildings 115 – 130 °C, the gap in the matrix 0.0042 – 0.005 m, the angular speed of the screw shaft 4 – 7 rad.s^{-1}, the time of technological influence on the oil-containing material in the tract of the press is 45 – 75 s.

Application of new working bodies allows to increase the productivity of the machine and to reduce energy consumption for the process of oil extraction. The theoretical compression ratio of a press extruder with a set of new working bodies for the processing of sunflower, rapeseed and soybean seeds is 4.69; 3.36 and 2.55. This ensured the intermediate compaction of the oil-containing raw material, the intensification of its crushing, and the increase of oil yield to 3%.



a) b)

Figure 4 Dependences of the force of grinding of raw materials by working cams F_P (a) and the cost of power when grinding products by working cams N (b) of the angular velocity of the drive shaft ω during the extrusion of oil from seeds. Note: 1 – sunflower (ρ = 440 kg.m^{-3}); 2 – rapeseed (ρ = 650 kg.m^{-3}); 3 – soybeans (ρ = 740 kg.m^{-3}).

CONCLUSION

1. Based on mathematical analysis of the movement of raw materials at separate stages of the extrusion process of oil in the developed twin-screw extruder determined the power and energy parameters of the research process and proved that the energy losses under the conditions of pre-grinding of seeds and, accordingly, reduction of technological resistance of the dispersed medium during oil extrusion are reduced by 2.52 times.
2. The graphical dependencies obtained in the MathCAD computer software environment showed that with an increase in the density of raw materials, it is grinded before being extruded by 40%, the power consumption for the extrusion process increases by 2.8 times and amounts to 0.7 – 2 kW for the recommended operating mode of the extruder.
3. For the recommended angular velocity of the extruder actuators ($\omega = 4 – 7$ rad.s^{-1}), the grinding force of the raw materials by the work rolls F_p increases by 3 times.

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DIFFERENCES BETWEEN MICROBIOTA, PHYTOCHEMICAL, ANTIOXIDANT PROFILE AND DNA FINGERPRINTING OF CABERNET SAUVIGNON GRAPE FROM SLOVAKIA AND MACEDONIA

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ABSTRACT

This study aimed to evaluate the microbiota, phytochemical, antioxidant profile and DNA fingerprinting of Cabernet Sauvignon grapes from Slovakia and R. North Macedonia. There were used two samples of grape berries (one sample from Slovakia and one from Macedonia). Each sample was analyzed in triplicate. The bacteria were cultivated on Plate count agar (PCA), microscopic filamentous fungi were cultivated on Malt extract agar (MEA). MALDI-TOF MS Biotyper mass spectrometry was used for the identification of microorganisms (bacteria and yeasts) and microscopic filamentous fungi with manuals. DPPH method was used to determine of antioxidant activity of grape berries. Phytochemical and antioxidant profiles were evaluated in grape berries samples. Total genomic DNA was extracted from mature grapes by GeneJET Plant Genomic DNA Purification Kit. The number of bacteria was higher in the sample of Macedonian grape (4.13 log CFU.g^{-1}) in comparison to the grape from Slovakia as well as the number of yeasts was also higher in the Macedonian sample (2.57 log CFU.g^{-1}). Antioxidant activity of Slovak grape berries was 0.55 mg TEAC.g^{-1} and of Macedonian grape, berries was 0.51 mg TEAC.g^{-1}. Total polyphenol content was higher in grape from Slovakia (0.81 mg GAE.g^{-1}) than in grape from Macedonia (0.77 mg GAE.g^{-1}), while total flavonoid content was 0.57 and 0.17 mg QE.g^{-1} in Slovak grape and Macedonian grape, respectively. Total phenolic acid content was higher in the sample from Macedonia (0.40 mg CAE.g^{-1}) compared to the grape from Slovakia (0.24 mg CAE.g^{-1}). Total anthocyanin content was also higher in Macedonian grape (0.46 mg.g^{-1}) compared to the Slovak sample (0.05 mg.g^{-1}). The total polymorphism for all of the used primers of 87.5% was obtained for the Macedonian sample of Cabernet Sauvignon and 89.4% for the Slovak sample.

Keywords: grape berries; bacteria; yeasts; antioxidant profile; MALDI-TOF MS Biotyper; polymorphism

INTRODUCTION

Grapes have been used for winemaking since the ancient Greek and Roman civilizations **(Ma and Zhang 2017)**. The presence of biologically active substances in fruits brings considerable benefits to consumers, whether consumed raw **(Durec et al., 2019)**. Grapes are rich in phytochemicals with many proven health benefits **(Liang et al., 2014)**. They are one of the most widely grown fruits and the total production of grapes worldwide is approximately 60 million tons **(Matthäus, 2008)**. Grapes can be categorized into grapes with edible seeds, seedless, wine grapes, table grapes, and raisin grapes **(Girard and Mazza, 1998)**. Grape seeds are rich in phenolic compounds and have potentially beneficial effects for human health such as protection against peptic ulcers, oxidative stress, tissue damage, and inflammation **(Rodríguez Montealegre et al., 2006; Kim et al., 2013)**. Grape seeds have been reported to exhibit scavenge superoxide radicals. Grape seeds are rich in flavan-3-ol, including proanthocyanidins and catechins **(El-Beshbishy, Mohamadin and Abdel-Naim, 2009)**.

Biological activities and health-promoting benefits are mostly possessed by polyphenols, which are considered to be the most important phytochemicals of grape. The phenolic compounds mainly include anthocyanins, flavanols, flavonols, stilbenes (resveratrol) and phenolic acids **(Xia et al., 2010)**.

From the vineyard to the winery, microorganisms play key roles in wine production and quality. The grapevine (*Vitis vinifera*) phyllosphere harbors diverse microbes including yeasts, filamentous fungi and bacteria that substantially modulate grapevine health, growth, and grape and wine production **(Gilbert, van der Lelie and Zarraonaindia, 2014)**.

Microbes could originate from the vineyard soil **(Morrison-Whittle and Goddard, 2018)**, air, precipitation (rainfall, hail, snow), be transported by animal vectors (bees, insects, and birds) **(Francesca et al.,**

2012; Stefanini et al., 2012; Lam and Howell, 2015), and be resident in nearby native forests **(Morrison-Whittle and Goddard, 2018)**.

Microbes that are grapevine-associated and are transferred to the must-have a profound influence on wine composition, flavor and quality **(Barata, Malfeito-Ferreira and Loureiro, 2012)**. Fermentative yeasts (primarily *Saccharomyces cerevisiae*) and lactic acid bacteria (LAB, predominantly *Oenococcus oeni*) in the must modulate the flavor and aroma of wine **(Swiegers et al., 2005)**.

In the study of **Kačániová et al. (2018)** a total of 33 species of 8 Gram-negative (20.72%), 10 Gram-positive (31.53%) bacteria and 10 yeasts species of 8 genera (47.74%) were identified with MALDI-TOF Mass Spectrometry.

Inter Primer Binding Site (iPBS) polymorphism is generated on the biological background of plant pararetroviruses, which primer binding site (PBS) is complementary to the 3′ end of the primer tRNA. In plant retrotransposons, the PBS is either complementary to the 3′ end or an internal region of the primer tRNA. The method of whole genome iPBS amplification is based on the virtually universal presence of a PBS in LTR retrotransposons **(Kalendar et al., 2010)**. This technique has been proved to be a powerful DNA fingerprinting technology without the need for prior sequence knowledge **(Fang-Yong and Ji-Hong 2014; Kalendar, Amenov and Daniyarov, 2018)**. It has the potential to discriminate among close genotypes **(Antonius-Klemola, Kalendar and Schulman, 2006)** and is highly reproducible **(Guo et al., 2014)**. Polymorphism generated by iPBS works well for both, the Gypsy and Copia LTR retrotransposons **(Melnikova et al., 2012)**.

Scientific hypothesis

Grape berries contain various microorganisms. Bacteria, yeasts and molds could be identified with MALDI TOF mass spectrometry.

There are many biologically active compounds in grape berries – flavonoids, polyphenols, phenolic acid and anthocyanins.

MATERIAL AND METHODOLOGY

Two types of grapes were studied in this work: one from Slovakia and one from Macedonia.

The phytochemical and antioxidant profile of the grape

The fresh grape berries were used for the preparation of ethanolic extract; 1 g of each sample was extracted with 20 mL of 80% ethanol for 2 h and centrifuged at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min. The supernatant was used for the measurement of antioxidant activity (DPPH) and the detection of total polyphenol, total flavonoid, and phenolic acid content.

Chemicals

All chemicals were of analytical grade and purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

DPPH Method—Radical Scavenging Activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the procedures described by **Sánchéz-Moreno, Larrauri and Saura-Calixto (1998)**. An amount of 0.4 mL of extract was added to 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). The absorbance of the reaction mixture was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Radical scavenging activity of the samples was expressed as Trolox equivalent antioxidant capacity (mg TEAC.g^{-1}).

Total Polyphenol Content

The total polyphenol content of extracts was measured by the method of **Singleton and Rossi (1965)** using Folin-Ciocalteu reagent. A 0.1 mL of each sample was mixed with 0.1 mL of Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate, and 8.8 mL of distilled water. After 30 min in darkness, the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid was used as the standard and the results were expressed in mg.g^{-1} of gallic acid equivalents.

Total Flavonoid Content

Total flavonoids were determined using the modified **Willett** method **(2002)**. A 0.5 mL of sample was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M potassium acetate, and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin was used as the standard and the results were expressed in mg.g^{-1} of quercetin equivalents.

Total Phenolic Acid Content

Total phenolic acid content was determined using a method of **Polish Pharmaceutical Society (2005)**. A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnow reagent (10% $NaNO_2$ + 10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid was used as a standard and the results were expressed in mg.g^{-1} of caffeic acid equivalents.

Total Anthocyanin Content

Anthocyanin content was measured according to the method of **Fuleki and Francis (1968)** with modifications **(Lee, Durst and Wrolstad, 2005)**. For pH 1.0, a sample (0.4 mL) was diluted with 0.025 M of potassium chloride (3.6 mL). For pH 4.5, a sample was diluted (0.4 mL) with 0.4 M of sodium acetate. The absorbance of the sample was measured at 520 and 700 nm against the blank reagent (distilled water). The concentration (mg.g^{-1}) of total anthocyanins was calculated according to the following formula and expressed as cyanidin-3-glucoside (Cy-3-glc) equivalent:

A [mg.g^{-1}] = (A∗Mw∗1000)/(ε∗L), A[mg.g^{-1}] = (A∗Mw∗1000)/(ε∗L),

where: A is the absorbance difference = ($A_{520} - A_{700}$) pH 1.0 − ($A_{520} - A_{700}$), pH 4.5; M_W is the molecular weight of (Cy-3-glc) = 449.2 g.mol^{-1}; ε is the extinction coefficient of (Cy-3-glc) = 1700 cm.mol^{-1}; L the absorption; path length : 1 cm.

Microbiological analyses of grape berries samples

Five grams of berries from each grape samples were diluted in 45 mL of sterile physiological saline (0.85%). Berries were stirred on a horizontal shaker for 30 minutes. After that, the dilutions of 10^{-2} and 10^{-3} were prepared for cultivation with the spread plate method. A 0.1 mL of each dilution (10^{-2}, 10^{-3}) was placed on the surface of a solid cultivation medium. Bacteria were cultivated on Plate count agar (PCA) (Oxoid, UK), yeasts on Malt extract agar base (MEA) (Oxoid, UK) supplemented with bromocresol green (0.020 g.L^{-1}) (Centralchem®, Slovakia). Bacteria were cultivated at 37 °C for 24 – 48 h in aerobic condition, yeasts at 25 °C for five days in aerobic conditions. Growing colonies with macroscopic morphological differences were recultivated on TSA (Tryptic Soy agar, Oxoid®). Inoculated plates were cultivated at 30 °C for 48 h (TSA). After cultivation, the proteins were extracted from fresh bacterial colonies.

Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial and yeast isolate was transferred into an Eppendorf vial and mixed in 300 µL of sterile water. After the addition of ethanol (900 µL), the suspension was mixed and centrifuged (13 000 g, 2 min). After removal of the supernatant, the pellets were dried at room temperature at least for 5 min. The bacterial and yeast pellets were resuspended in 20 – 50 µL of formic acid (70%) and the same amount of acetonitrile. After centrifugation (2 min at 13,000 g), a 1 µL of supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. A 1 µL of MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot and dried.

The MALDI target plate was introduced into the MALDI-TOF mass spectrometer (Bruker, Germany) for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, log(score) ≥2.0 was set as the threshold for a match at the species level. Isolates with ≥2.0 were accepted as a correct identification.

DNA extraction and iPBS profiles amplification

Total genomic DNA was extracted from mature grapes by GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher) following the instructions of the manufacturer. The iPBS primers 1845, 1846 and 1886 were used for the fingerprints amplification **(Kalendar et al., 2010)**. The following iPBS PCR profile was used for the Combi PPP 2x MasterMix (Top Bio) and 50 ng of DNA: 94 °C – 5 min; 35 cycles of : 95 °C 1 minute; 55 °C 2 minutes; 72 °C 3 minutes with final 72 °C 10 minutes. Amplified fragments were analyzed in 6% PAGE and scored for the presence or absence of amplicons in GelAnalyser software. UPGMA analysis and dendrogram construction was performed in SYNTAX software using a Jaccard coefficient of similarity to define relationships between individual obtained iPBS profiles for analyzed samples of Cabernet Sauvignon.

Statistical analysis

All experiments were carried out in triplicate and standard deviations for replication as well as T-tests were calculated using MS Excel.

RESULTS AND DISCUSSION

The phytochemical and antioxidant profile of studied grapes (or grape samples)

According to many authors, the antioxidant activity of grape berries and wines results mainly from their phenolics, whereas the phenolic content and composition depend on the grape variety, vineyard location, cultivation system, climate, soil types, vine cultivation practices, harvesting time, production process and aging **(Shahidi and Naczk, 1995).**

DPPH·method is one of the most popular methods for detecting the antioxidant activity of wine **(Wang, 2008)**. The experimental results indicate that the higher the amount of antioxidants, the lower is the concentration of remaining DPPH and the stronger is the radical-scavenging activity **(Jiang and Sun, 2018)**.

The antioxidant activity of Slovak grape berries was 0.55 mg TEAC.g^{-1} and antioxidant activity of Macedonian grape were 0.51 mg TEAC.g^{-1}. **Jiang and Zhang (2012)** reported that the contents of phenolic compounds and the levels of antioxidant activity in the wine samples greatly varied with cultivar and environmental factors of wine growth.

The value of total polyphenols was 0.81 mg GAE.g^{-1} in grape from Slovakia and 0.77 mg GAE.g^{-1} in grape berries from Macedonia. Total flavonoids were 0.57 mg and 0.17 QE.g^{-1} in Slovak and Macedonian grape berries, respectively. Phenolic compounds, which are abundant in grape berries and wines, play one of the most important roles in the quality of grapes and wines. They strongly contribute to the color, mouthfeel and palatability of red wines **(Lesschaeve and Noble, 2005)**. Moreover, polyphenols also exert many favorable effects on human health, such as the inhibition of atherosclerosis, coronary heart disease, and various cancer types **(Yilmaz and Toledo, 2004)**. Total phenolic acid content was 0.24 and 0.40 mg CAE.g^{-1} in grape from Slovakia and grape from Macedonia, respectively.

Table 1 Antioxidant activity, total polyphenol, flavonoid, phenolic acid and anthocyanin content of analyzed grape.

Samples	DPPH mg TEAC.g^{-1}	TPC mg GAE.g^{-1}	TFC mg QE.g^{-1}	TPAC mg CAE.g^{-1}	TAC mg.g^{-1}
Slovak Cabernet Sauvignon grape	0.55 ±0.01	0.81 ±0.05	0.57 ±0.05	0.24 ±0.07	0.05 ±0.01[a]
Macedonia Cabernet Sauvignon grape	0.51 ±0.15	0.77 ±0.09	0.17 ±0.02	0.40 ±0.01	0.46 ±0.03[a]

Note: *DPPH* - 2,2-difenyl-1-picrylhydrazyl *TPC* total polyphenol content, *TFC* total flavonoid content, *TPAC* total phenolic acid content, *TAC* total anthocyanin content, *GAE* gallic acid equivalent, *QE* quercetin equivalent, *CAE* caffeic acid equivalent, *FM* fresh matter; [a] significant difference of analysed parameter.

Table 2 Microorganisms counts isolated from wine grapes in log CFU.g^{-1}.

Sample	Bacteria	Yeasts
Slovak Cabernet Sauvignon grape	3.57 ±0.29	2.34 ±0.27
Macedonian Cabernet Sauvignon grape	4.13 ±0.08	2.57 ±0.18

Table 3 Microorganisms isolated from wine grape berries.

Slovak Cabernet Sauvignon grape	*Alternaria* sp., *Bacillus endophyticus, Escherichia coli, Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Pantoea agglomerans, Stenotrophomonas maltophilia, Yarrowia lipolytica, Botrytis cinerea, Cladosporium* sp., *Ignatzschineria indica, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus paracasei, Penicillium expansum*
Macedonian Cabernet Sauvignon grape	*Alternaria* sp., *Bacillus endophyticus, Escherichia coli, Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Lactobacillus fermentum, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Pantoea agglomerans, Stenotrophomonas maltophilia, Yarrowia lipolytica, Bacillus cereus, Issatchenkia orientalis, Lactobacillus paracasei*

Macedonian Cabernet-Savignon			Slovak Cabernet-Savignon		
1845 iPBS profile	1846 iPBS profile	1886 iPBS profile	1845 iPBS profile	1846 iPBS profile	1886 iPBS profile
1	1	0	1	0	0
0	0	1	0	0	1
0	0	1	1	0	0
1	0	0	0	0	0
0	1	0	1	1	0
1	1	0	0	0	0
1	0	0	0	0	1
0	0	0	1	0	0
0	0	0	1	0	0
1	1	1	1	1	0
1	0	0	1	0	1
0	0	1	1	0	1
1	1	0	1	1	0
0	0	0	0	0	1
1	0	0	1	0	0
1	1	1	1	1	1
0	0	0	1	0	0
0	1	1	1	1	0
0	1	1	1	0	1
0	0	0	1	0	0
1	1	1	1	1	1

Figure 1 Amplification profiles of analysed samples of Cabernet Sauvignon.

Yang, Martinson and Liu (2009) analyzed the total phenolic contents of 14 wine grapes. Among all the grape varieties analyzed, Cabernet Franc and Pinot Noir had the highest total phenolic content (424.6 ±3.8 and 396.8 ±12.4 mg of gallic acid equivalents per 100 g of grape, respectively), followed by Concord, Sheridan, Chancellor, Marechal Foch, Catawba, DeChaunac, Riesling, Niagara, Vidal Blanc, Baco Noir, Cayuga White, and Chardonnay.

Mitić et al. (2012) measured the total flavonoid content of the 7 grape extracts, 'Cabernet Sauvignon' presented the highest flavonoid content (146.2 mg of CE.100 g^{-1} of f.w.), followed by 'Merlot', 'Vranac', 'Muscat Hamburg', 'Prokupac', 'Ribier', and 'Cardinal'. **Ivanova, Stefova and Chinnici (2010)** measured lower values 60.3 CE.100g^{-1} f.w. the average total flavonoids content of grape cultivars 'Vranec', 'Cabernet Sauvignon', and 'Muscat Hamburg'.

Anthocyanins are natural pigments, responsible for a wide range of colors in grapes and red wines. The anthocyanins in red grapes vary greatly with the species, maturity, production area, seasonal conditions, and yield of the fruit **(Mitić et al., 2012)**. The total anthocyanin content was 0.05 mg.g^{-1} in grape from Slovakia and 0.46 mg.g^{-1} in grape from Macedonia. Table 1 compares data related to antioxidant activity, total polyphenol, flavonoid, phenolic acid and anthocyanin content of analyzed grape. The statistical difference was found only in TAC.

Microbiota of grape

The surface of grape berries represents a comprehensive natural reservoir of bacterial microbiota originating from the surrounding environment **(Zarraonaindia et al., 2015)**. The value of bacteria was 3.57 log CFU.g^{-1} in Slovak grape and 4.13 log CFU.g^{-1} in Macedonian grape berries. The value of yeasts was 2.34 log CFU.g^{-1} in Slovak grape and 2.57 log CFU.g^{-1} in Macedonian grape (Table 2). Numerous yeast genera and species are found during the production of wine. The low pH of the wine, high sugar content, rapidly generated anaerobic conditions, and presence of phenolic compounds create the ideal environment to support the growth of yeasts and to enrich these organisms with other microbes **(Fleet, 2003)**.

Grapes have a complex microbial ecology including filamentous fungi, yeasts, and bacteria with different physiological characteristics and effects upon wine production. Some species are only found in grapes, such as parasitic fungi and environmental bacteria, while others can survive and grow in wines, constituting the wine microbial consortium. This consortium covers yeast species, lactic acid bacteria, and acetic acid bacteria **(Barata, Malfeito-Ferreira and Loureiro, 2012).**

Bacterial populations are usually several orders of magnitude lower than those of yeasts in sound grapes. Lactic acid bacteria have counts lower than 10^2 CFU.g^{-1} **(Francesca et al., 2011)**.

Table 3 presents microorganisms isolated from Slovak and Macedonian grape berries. Worldwide surveys indicate that sound grapes are colonized by a wide variety of yeast species without any obvious explanation. However, the variety may be reduced to relatively few groups of similar physiological characteristics. For instance, the ubiquitous *Candida* spp. and *Pichia* spp. are highly heterogeneous, and new species are likely to be found in each new survey due to the accuracy of molecular identifications is constantly increasing **(Rao et al., 2007)**.

Grapevine bacteria play a key role not only in plant health but also in crop quality and yields, which can influence the winemaking process **(Nisiotou et al., 2011)**. Numerous studies have analyzed the presence of yeast on the surface of grapes and many have indicated that *Saccharomyces cerevisiae* is only present in small numbers on healthy grapes **(Pretorius, 2000)**. *Saccharomyces* can be found in grape musts, but the populations are often less than 50 CFU.mL^{-1} **(König, Unden and Fröhlich, 2009)**. We did not isolate *Saccharomyces cerevisiae* in our study.

The yeast populations of grapes generally comprise between 10^2 and 10^4 cells.g^{-1} **(Fleet et al., 2002)**, but higher values have also been reported. *Hanseniaspora uvarum* appears to be the most common grape berry species worldwide, which is consistent with its predominance at the beginning of spontaneous must fermentations. Like yeasts, lactic acid bacteria are also found in vineyards **(Lonvaud-Funel, 1999)**.

The microbiota of grapes also includes fungi that may dominate under favorable weather conditions accompanied by inefficient phytochemical utilization. Fungal obligate parasites can penetrate through the intact grape skin by their own biochemical and mechanical activities and are responsible for high economic losses. The main species are the oomycete *Plasmopara viticola,* responsible for downy mildew, and the ascomycetes *Erysiphe necator* (powdery mildew), *Elsinoë ampelina* (anthracnose), *Guignardia bidwellii* (black rot) and *Pseudopezicula tracheiphila* (rotbrenner) **(Barata, Malfeito-Ferreira and Loureiro, 2012)**.

DNA fingerprinting

The variability in polymorphism length was inspected among the Macedonian and Slovak Cabernet Sauvignon grapes using an iPBS markers 1845, 1846 and 1886. The total number of obtained iPBS fragments was 57 which were distributed to 21 levels. The average number of fragments per primer was 9.5. Their size ranged from 378 bp up to the 882 bp. The level of the shortest fragments was present in both of the analyzed varieties for all of the used primers (Figure 1). The highest number of obtained fragments per one primer was 16 fragments for Cabernet Sauvignon from Slovak growing conditions analyzed by primer 1845. The lowest number – 6 fragments for Cabernet Sauvignon from Slovak growing conditions analyzed by primer 1846. One unique fragment was amplified for the Cabernet-Sauvignon from Macedonian growing conditions analyzed by primer 1845 with the length 787 bp.

The total polymorphism for all of the used primers of 87.5% was obtained for the Macedonian sample of Cabernet Sauvignon and 89.4% for the Slovak sample. The most similar iPBS profiles of Slovak and Macedonian samples of Cabernet Sauvignon grapes were obtained for the primer 1846 (Figure 2).

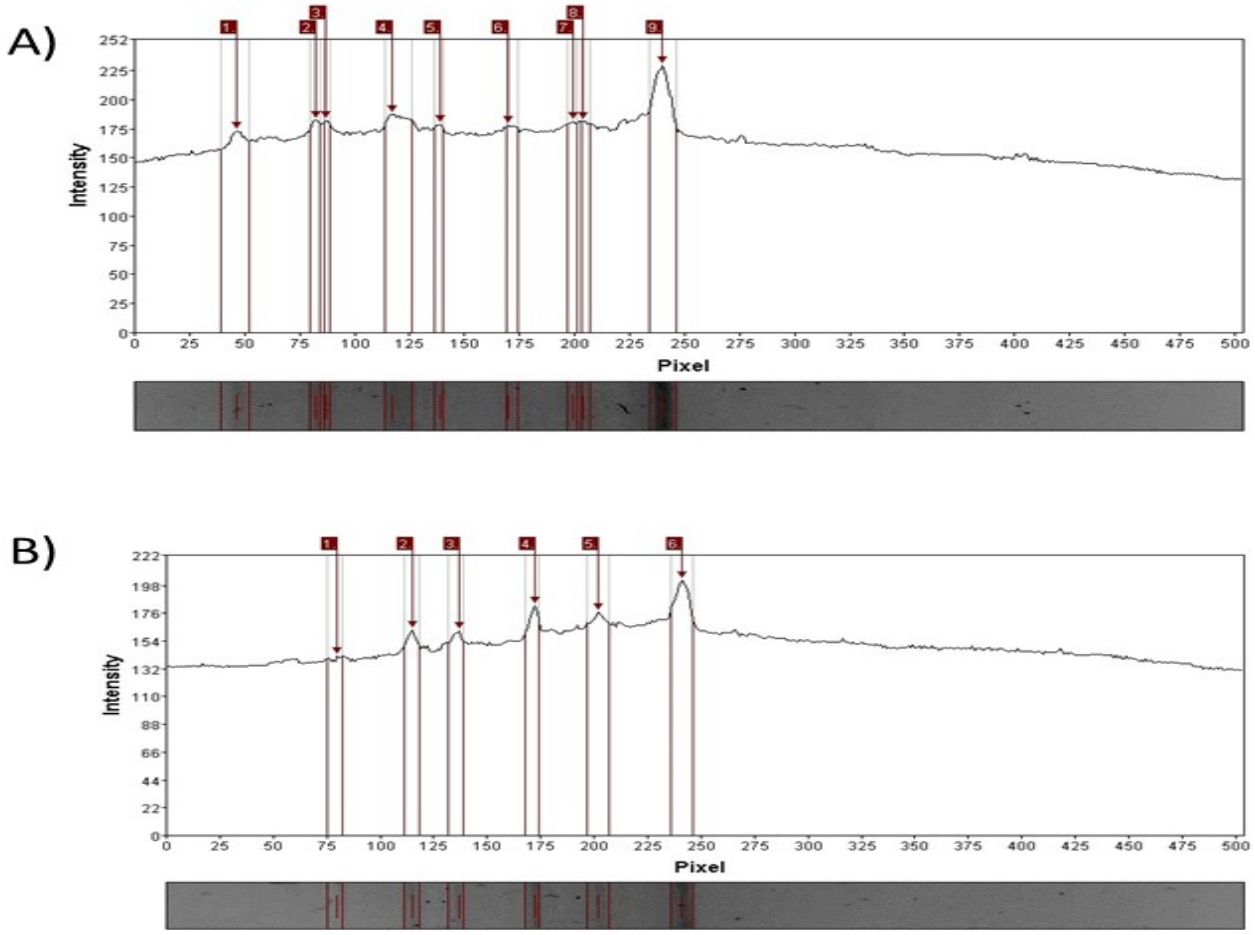

Figure 2 Analysis of length of obtained 1846 fragments for samples from Moldavia(A) and Slovakia (B) evaluated by software GelAnalyzer.

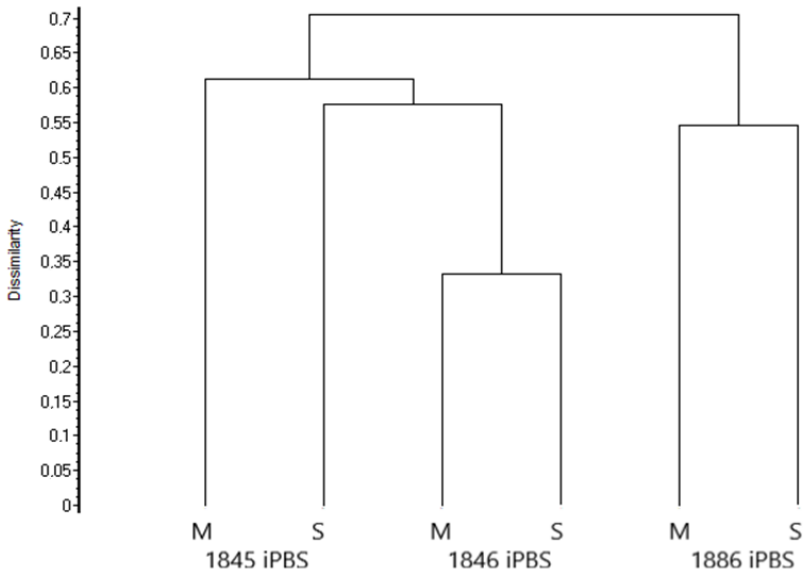

Figure 3 Dendrogram of obtained iPBS profiles of analysed samples of Cabernet-Sauvignon.

The analysis of the relationships of obtained iPBS amplicon profiles was performed by the clustering method using the UPGMA analysis (Figure 3). A clear effect of primer can be seen preferentially to the provenience of the analyzed samples. Profiles generated by primers 1845 and 1846 were grouped and the profiles of 1886 primer were separated as a new branch at the level of 0.7. Here again, the highest similarity of 1846 iPBS profiles of Macedonian and Slovak samples was confirmed.

The PBS primed PCR generated markers are reported to be very effective for extensive intraspecific polymorphism detecting, including in the study of clonal variability. Genotyping by iPBS markers was used for finding differences between varieties and their clones as well as one of the tools for grapevine collection management **(Butorac et al., 2018; Drori et al., 2017)**. Shannon index with the value of 0.137 was reported for Cabernet Sauvignon for six genotypes by **Milovanov et al. (2019)** when a total of 30 PBS primers were used.

CONCLUSION

In our study, the Slovak grape berries sample contained a higher concentration of polyphenols and flavonoids, but a lower concentration of phenolic acids and anthocyanins in comparison to the Macedonian grape. The number of yeasts and bacteria was higher in grape berries from Macedonia. Weather and cultivation conditions can affect the content of biologically active components as well as microorganisms in grape berries.

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FLOW CYTOMETRY AS A RAPID TEST FOR DETECTION OF TETRACYCLINE RESISTANCE DIRECTLY IN BACTERIAL CELLS IN *MICROCOCCUS LUTEUS*

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ABSTRACT

Correct effective doses of antibiotics are important in the treatment of infectious diseases. The most frequently used methods for determination of the antibiotic susceptibility of bacterial pathogens are slow. The detection of multidrug-resistant bacteria currently relies on primary isolation followed by phenotypic detection of antibiotic resistance by measuring bacterial growth in the presence of the antibiotic being tested. The basic requirements for methods of detection of resistance to antibiotics include speed and accuracy. We studied the speed and accuracy of flow cytometry for the detection of tetracycline resistance in the Gram-positive bacteria *Micrococcus luteus*. Detection of cell viability and reliability of antibiotic resistance was carried out on the Guava EasyCyte flow cytometer (Merck Millipore, Germany) with SYBR Green and PI dyes. *M. luteus* was exposed to tetracycline (at 30, 90, 180 and 270 μg.mL^{-1}) over 24 hours. Concentrations of live and dead cells were measured after 4 and 24 hours of incubation. The results revealed that the use of mixed dyes PI and SYBR Green allowed the division of cells into large subpopulations of live and dead cells and the DNA of destroyed cells. After 4 h exposure to tetracycline 30 μg.mL^{-1}, the subpopulation of live cells decreased by 47% compared to the positive control. Tetracycline at 90 μg.mL^{-1} decreased the subpopulation of live cells by 59% compared to the positive control. A continued increase in concentration caused a shift in the population and an increase in dead cells, indicating damage to the cells of the microorganism. Incubation of M. *luteus* with 180 and 270 μg.mL^{-1} tetracycline decreased the subpopulation of live cells by 82% and 94%, respectively, in comparison with the positive control. After incubation with 30 μg of tetracycline over 24 h the number of living cells decreased by 70% in comparison with the positive control. Tetracycline treatment (90 μg.mL^{-1} for 24 h) killed 71% of cells. After exposure to 90 μg.mL^{-1} tetracycline 29% cells were viable. The viability of living cells was confirmed by a microbiological test.

Keywords: antibiotics; flow cytometry; antibiotic resistance; Micrococcus luteus

INTRODUCTION

Antibiotics are one of the most beneficial discoveries in medicine and public health. However, the use, overuse and misuse of these drugs have led to increases in antibiotic-resistant bacterial infections. Antimicrobial resistance (AMR) poses a serious global threat of growing concern to human, animal and environmental health. This is due to the emergence, spread, and persistence of multidrug-resistant bacteria **(Davies and Davies, 2010)**. The O'Neill Review on Antimicrobial Resistance estimated that 700,000 people die from infections due to resistant organisms every year, and by 2050 AMR will surpass cancer as a cause of death **(O'Neill, 2016)**. Resistance to so-called critically important antibiotics used in medicine is of special concern **(Bataeva and Zaiko, 2016)**.

Fast, accurate antibiotic susceptibility testing is a critical need in addressing escalating antibiotic resistance, since delays in identifying multidrug-resistant organisms increase mortality **(Kadri et al., 2018)**.

Standard methods of detection of antibiotic sensitivity are labor- and time-consuming. Detection of multidrug-resistant bacteria currently relies on primary isolation followed by the phenotypic detection of antibiotic resistance by measuring bacterial growth in the presence of the antibiotic being tested. These conventional methods take a minimum of 24 hours to obtain results after the pure culture is isolated (the analysis typically lasts up to 72 hours) **(Akhmaltdinova, Lavrinenko and Belyayev, 2017)**. Working out express diagnostic methods is of importance, and currently, studies are made in various directions **(Wang et al., 2010)**.

One of these directions is the use of flow cytometry (FC) for the detection of microorganism viability and resistance. Flow cytometry was adopted for microbiological purposes almost 40 years ago, and the usefulness of this method for

the identification of microbial pathogens directly in clinical samples or the detection of specific antibodies in serum has been well studied **(Álvarez-Barrientos et al., 2000)**, as has its use in the study of antimicrobial activity of some animal-generated antimicrobial substances **(Kotenkova and Polishchuk, 2019)**.

The quantitative assessment of prokaryotic viability is essential, especially for the confirmation of the activity of novel antimicrobial substances **(Kotenkova et al., 2019)**. Flow cytometry can be used for the analysis of the individual population of cells; therefore, it can provide essential information about bacterial antibiotic resistance. Recently, investigations of bacterial antibiotic resistance seem to be most relevant in the clinical environment because of the problems in finding effective therapies. In comparison with traditional diagnostic methods, FC allows results to be obtained much more quickly **(Álvarez-Barrientos et al., 2000)**. Currently, there are reported experiences of antimicrobial susceptibility tests by FC **(Woźniak-Kosek and Kawiak, 2005; Faria-Ramos et al., 2013)**, but despite the significant progress of clinically significant protocols of FC application, there is insufficient scientific information on its continuous use in microbiology.

This manuscript reports the results of the use of FC with the dyes SYBR Green and PI for rapid assessment of cell viability and antibiotic resistance of the Gram-positive bacteria *Micrococcus luteus*.

Scientific hypothesis

There is a considerable need for new techniques that enable quick and specific diagnosis of pathogens resistant to antibiotics to guide correct treatment and to slow the development of resistance. Flow cytometry can provide quick essential information about the resistance to antibiotics of pathogenic microorganisms.

MATERIAL AND METHODOLOGY

Micrococcus luteus ATCC 4698 from American Type Culture Collection was used.

Sample preparation

Positive, negative and mixed controls of Micrococcus luteus ATCC 4698

Micrococcus luteus ATCC 4698 strain was obtained from the State Research Center for Applied Biotechnology and Microbiology (Obolensk, Moscow region, Russia). Cultures were grown on slanted Trypticase soy agar (TSA, Liofilchem) at 30 °C for 24 h. Cultures from the agar surface were removed with Trypticase soy broth (TSB, Liofilchem) and incubated at 30 °C for 24 h. Cultures were grown to exponential phase (OD_{600} 0.3). The received suspension was used as a positive control.

To obtain negative control, 1 mL suspension was heated at 100 °C for 10 min. To prepare mixed samples, 200 µL of positive control (live cells) and 200 µL of a negative control (dead cells killed by heating) were used.

Preparation of antibiotics

Cultures were grown to early exponential phase, at which time tetracycline (Sigma-Aldrich, USA) was added in various concentrations (30 $\mu g.mL^{-1}$, 90 $\mu g.mL^{-1}$, 180 $\mu g.mL^{-1}$ and 240 $\mu g.mL^{-1}$). Incubation was carried out at 37 °C for 24 h. Concentrations of live and dead cells were measured after 4 and 24 h of incubation with the antibiotic.

Flow cytometry analysis protocol

SYBR Green stock solution was prepared by dissolving 5 µL of SYBR ™ Green I Nucleic Acid Gel Stain, 10,000 X concentrate in DMSO (Thermo, USA) in 495 µL of deionized water. A PI working solution was prepared by dissolving 1 mg PI (AppliChem, Germany) in 1 mL of deionized water immediately before the study.

A volume of 975 µL of 1X pH 7.4 PBS (Santa Cruz, USA) was mixed with 10 µL of SYBR Green (Thermo, USA) and 5 µL PI (AppliChem, Germany), shaken vigorously, then 10 µL of *M. luteus* ATCC 4698 was added and mixed. The samples were incubated in the dark for 15 min and green and red fluorescence signals measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) for up to 5000 events.

Microbiology test

Verification of cell viability was obtained by microbiological test by transferring 100 µL of each concentration onto a nutrient agar plate (TSA, Liofilchem). All plates were incubated for 3 – 5 days at 30 °C. The growth on nutrient agar was evaluated by the presence of viable cells in the sample.

Statistical analysis

Measurements were repeated three times. STATISTICA 10.0 software was used for statistical analyses. The results were calculated as mean ± standard deviation (M ±*SD*). Significant differences (comparison with positive control) were tested by one-way ANOVA *Dunnet* test. Differences with *p*-values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Routine techniques for the detection of resistance to antibiotics are based on a phenotypic study in which microbial growth is observed in the presence of different antibiotics. They yield results in not less than 24 h. In the last two decades, faster AST methods, such as PCR-based tests **(Barken et al., 2007)** and mass spectrometry-based methods **(Opota et al., 2015; Trip et al., 2015)** have been developed. However, they do not always provide relevant information on antibiotic susceptibility. For example, PCR-based tests rely on the detection of resistance mutations and genes. However, bacteria lacking resistance mutations and genes may still be able to tolerate and survive antibiotic treatments by utilizing many other mechanisms, some of which are non-genetic **(Javid et al., 2014; Sanchez-Romero and Casadesus, 2014)**. Changes in bacterial physiology caused by antibiotics can be detected using FC and fluorescent viability markers, as has been demonstrated by numerous studies **(Gant et al., 1993; Walberg et al., 1997; Gauthier et al., 2002; Ambriz-Avina et al., 2014)**.

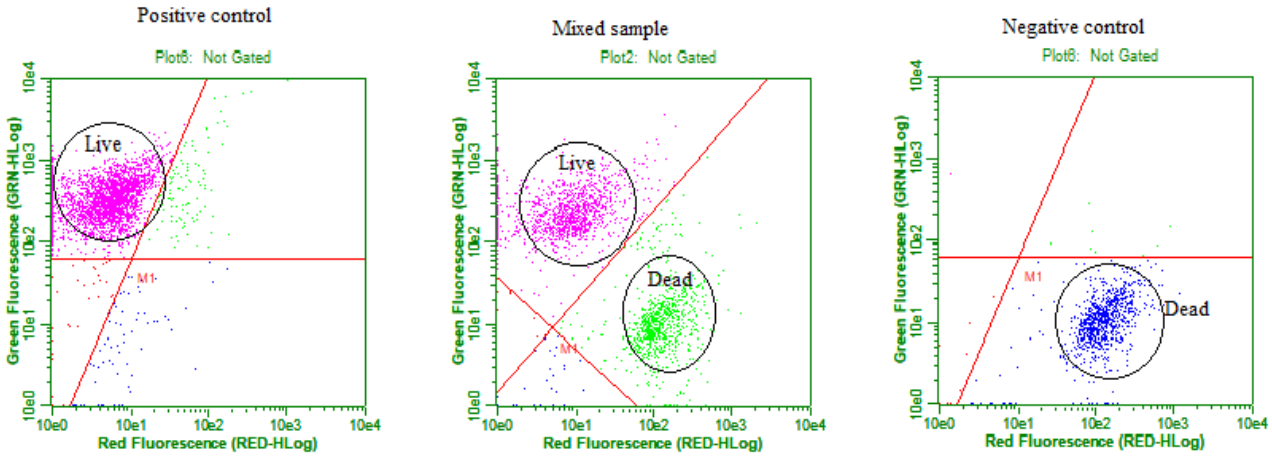

Figure 1 Result of flow cytometry analysis of positive, negative controls and mixed sample of *Micrococcus luteus* ATCC 4698 without antibotic influence .

M. luteus was the object of this study. It is a Gram-positive microorganism with cell walls consisting of two polymers, i.e., peptidoglycans **(Schleifer and Kandler, 1967)** and teichuronic acids (TUAs) **(Hase and Matsushima, 1972)**.

M. luteus can form dormant structures that allow for increased survival under stress. So, cold (4 °C), dryness (2.5% humidity) and starvation increase the survival of *M. luteus* **(Casida, 1980)**, possibly by creating unfavorable growth conditions, and thereby inducing dormancy **(Dib et al., 2013)**. Thus, cells can survive long periods under adverse environmental conditions **(Kaprelyants and Kell, 1993)**, which can allow the evaluation of the gradual transition of cells from living to die over time. PI and SYBR Green dyes can identify stained cells in different large subpopulations. Resistant organisms are more difficult to kill than susceptible organisms, and the SYBR Green/PI assay can distinguish resistant and susceptible categories based on the number of residual viable cells after drug treatment **(Feng et al., 2014)**.

Figure 1 presents the staining patterns of *M. luteus* control samples. Positive control *M. luteus* ATCC 4698 (live cells) displayed green fluorescence, and the corresponding counted events are located in the upper left square of the plot. Negative control (dead cells) displayed red fluorescence, and all counted events are located in the lower right square of the plot.

After 4 h exposure of *M. luteus* to tetracycline (at 30 µg.mL^{-1}, 90 µg.mL^{-1}, 180 µg.mL^{-1} and 270 µg.mL^{-1}) the mix of dyes identified the emergence of large subpopulations of live and dead cells and the DNA of destroyed cells (Figure 2).

In Figure 2 the red marker corresponds to live cells, blue to dead cells, and purple to the DNA of destroyed cells. There is an intensification in the displacement of the population of living cells beyond the red marker towards the blue, which indicates dead cells. Since PI and SYBR Green are DNA-binding dyes, it is possible to see a third population on the cytogram – the DNA of destroyed cells. The increase in the number of cells inside the blue marker shows a rapid decline in the numbers of viable bacteria after the addition of antibiotics.

Results of the FC analysis of *M. luteus* after 24 h incubation with various concentrations of tetracycline are presented in Figure 3. The difference in relative mean fluorescence intensity between live and dead cells increased further following 24 h of treatment. A further decrease in the number of living cells from the initially treated cells was noted, and the presence of a large amount of debris indicated that cell lysis was occurring.

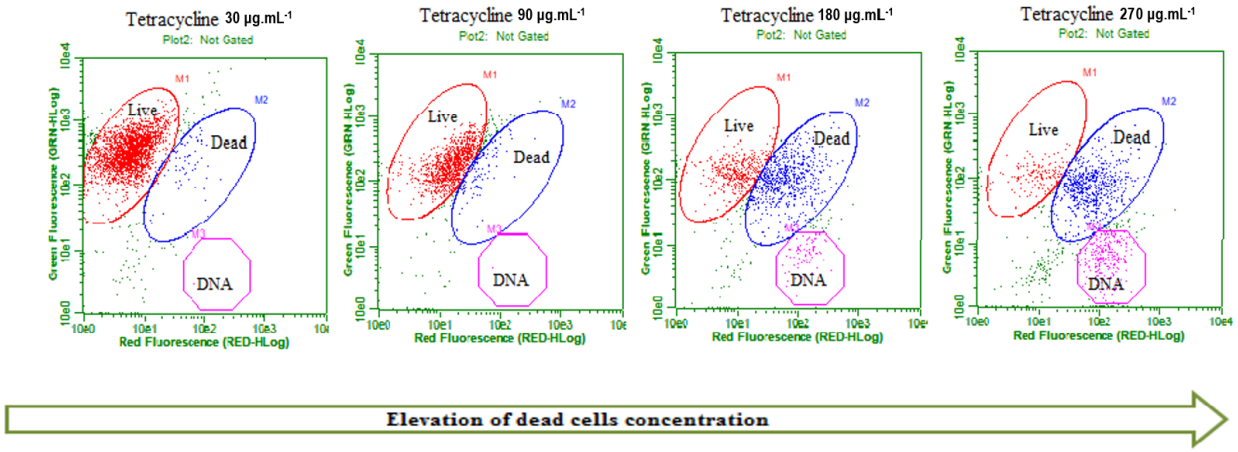

Figure 2 Results of flow cytometry after exposure of *M. luteus* to tetracycline (at 30 µg.mL^{-1}, 90 µg.mL^{-1}, 180 µg.mL^{-1} and 270 µg.mL^{-1}) during 4 h incubation.

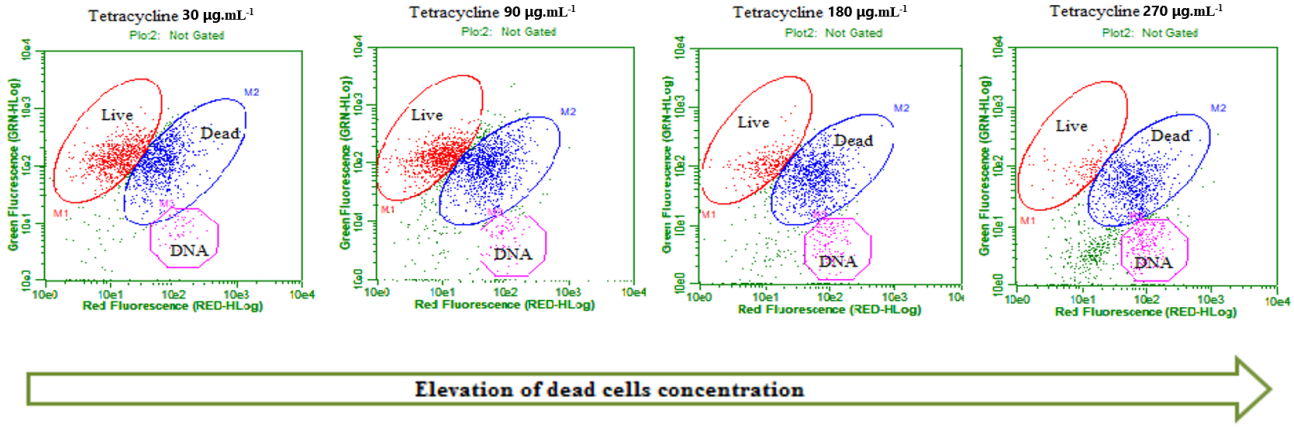

Figure 3 Results of flow cytometry after exposure of *M. luteus* to tetracycline (at 30 µg.mL^{-1}, 90 µg.mL^{-1}, 180 µg.mL^{-1} and 270 µg.mL^{-1}) during 24 h incubation.

After 4 h exposure to tetracycline at 30 µg.mL^{-1}, the subpopulation live cells decreased by 47.0% compared to the positive control (Table 1). Tetracycline solution at 90 µg.mL^{-1} decreased the subpopulation of live cells by 59.0% compared to the positive control.

A continued increase in concentration caused a shift in the population and an increase in dead cells, indicating damage to the cells of the microorganism. So, incubation of *M. luteus* with 180 µg.mL^{-1} and 270 µg.mL^{-1} tetracycline decreased the subpopulation of live cells by 82.0% and 94%, respectively, in comparison with the positive control. The viability of living cells was confirmed by a microbiological test at all concentrations of the antibiotic.

This completely coincided with the results of the cytometric analysis. In analogical research, FC was used for detecting resistant *E. coli* strains. Cytometry showed acceptable results on the model of *E. coli*. Relative accuracy was 88.8%, sensitivity – 85.7%, specificity 88.8%, and Cohen's kappa test showed a value of 0.524 **(Akhmaltdinova, Lavrinenko and Belyayev, 2017)**.

Cytometry analysis showed a decline in the numbers of viable bacteria after 24 h incubation of *M. luteus* with antibiotics. Incubation with 30 µg of tetracycline decreased the number of stained living cells by 70% in comparison with the positive control. Tetracycline treatment at 90 µg.mL^{-1} for 24 h killed 71.0% of cells. So, after exposure to 90 µg.mL^{-1} tetracycline, 29.0% of cells were vaible.

The viability of living cells was confirmed by a microbiological test. Incubation on the plate was increased to 5 days. A study by **Mukamolova et al. (1998)** reported that the viability of *M. luteus* cells was restored after some 96 h incubation of starved cells in a resuscitation medium.

After 24 h exposure to tetracycline (180 µg.mL^{-1} and 270 µg.mL^{-1}) the majority of the cells in the population had received antibiotic-induced damage. The number of stained living cells decreased by 89% and 98.9%, respectively, in comparison with the positive control **(Mukamolova, Kaprelyants and Kell, 1998)**. The viability of the remaining living cells was confirmed only at a tetracycline concentration of 180 µg.mL^{-1}. Such cell survival can be explained by the ability to transition to cell dormancy in unfavourable growth conditions **(Dib et al. 2013)**. In the study by **Nikitushkin et al. (2016)**, it was reported that in response to unfavourable growth conditions nonsporulating mycobacteria transform into the dormant state with the concomitant formation of specialized dormant forms characterized by low metabolic activity and resistance to antibiotics. This because *M. luteus* secretes a small protein called Rpf, which has autocrine and paracrine signaling functions and is required for the resuscitation of dormant cells **(Nikitushkin, Demina and Kaprelyants, 2016)**.

Dormancy is a protective state that enables bacteria to survive antibiotics, starvation and the immune system. Dormancy comprises different states, including persistent and viable but nonculturable (VBNC) states that contribute to the spread of bacterial infections **(Mali et al., 2017)**.

Food products are a source of antibiotic-resistant pathogenic bacteria, a way for the transmission of antibiotic-resistant 'food' pathogens through the food chain to humans **(Zaiko et al., 2019)**. There is an ongoing increasing antibiotic resistance crisis and new drugs and antibiotics are urgently needed to combat life-threatening antibiotic-resistant infections **(Feng et al., 2018)**.

Table 1 The results of measuring the number of cells by flow cytometry.

Time incubation	K «+»			Tetracycline											
				30 µg.mL^{-1}			90 µg.mL^{-1}			180 µg.mL^{-1}			270 µg.mL^{-1}		
	*10^7 cells.mL^{-1}														
	total	live	dead	total	live	dead	total	live	dead	total	live	dead	total	live	dead
4 hours	5.34^{a}	5.20	0.14	3.07^{b,c}	2.80	0.27	2.84^{b}	2.10	0.74	2.40^{b}	0.90	1.5	1.50^{b,d}	0.30	1.20
24 hours	22.28^{a}	22.00	0.28	3.30^{b,c}	1.60	1.70	3.40^{b}	1.50	1.90	2.58^{b}	0.58	2.0	2.06^{b,d}	0.26	1.80

Note: * – significant differences of experimental (tetracycline) doses compared with positive control (p <0.05).

Fast and accurate antibiotic susceptibility tests can significantly reduce mortality rates and reduce financial costs **(Barenfanger et al., 1999)**. Furthermore, the necessity of rapidly prescribing an initial empirical antimicrobial treatment while waiting for the susceptibility test results from time-consuming standard methods frequently leads to inappropriate treatments **(Ibrahim et al., 2000)**.

As to future work, the same strategy may usefully be applied to other microorganisms (including pathogens in difficult food matrixes and other antibiotics. However, the present work shows that one may indeed expect to be able to determine antibiotic susceptibility by FC methods. This could be a very useful tool in the fight against antimicrobial resistance.

CONCLUSIONS

The current conventional methods for the determination of minimal inhibitory concentration (MIC) rely on the growth of the test organism in the presence of the antibiotic, which can be time-consuming depending on the growth speed of the organism, ranging from days for fast-growing bacteria to weeks for slow-growing bacteria. We reported a rapid and novel antibiotic susceptibility testing methodology using FC. This study to demonstrate the feasibility of FC used SYBR Green/PI dyes to assay rapidly the viability of cells and to detect reliably the antibiotic resistance of the Gram-positive bacteria *Micrococcus luteus*.

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THE EFFECT OF FORTIFIED *DADIH* (FERMENTED BUFFALO MILK) WITH VITAMIN D_3 ON CAECUM CHOLESTEROL CONCENTRATION AND HIGH SENSITIVITY C-REACTIVE PROTEIN (hs-CRP) LEVEL IN TYPE 2 DIABETES MELLITUS RAT MODEL

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ABSTRACT
Type 2 diabetes mellitus (T2DM) may be developed by a cardiovascular complication. T2DM and its complications associated with a decrease in levels of 25 hydroxyvitamin D below normal. The level of 25-OH-D can increase and it can be gained by probiotics supplementation. *Dadih* is a probiotic useful as antidiabetic, antiatherosclerotic, and it can reduce serum cholesterol. Vitamin D is beneficial for T2DM since it improves insulin production, acts as an anti-inflammatory and prevents dyslipidemia thereby preventing cardiovascular disease. This research aims to investigate the effects of giving *dadih*-fortified-vitamin-D_3 toward caecum-cholesterol-concentration and hs-CRP-levels to T2DM-induced-rats. This study used a randomized pre-post test with control group design in 30 Wistar rats divided into 5 groups, namely T1, T2, and T3-treatment-groups. T3-group was given *dadih*-fortified-vitamin-D_3, while T1 and T2-groups were given vitamin-D_3 and *dadih*, respectively. The control groups were healthy-control (C1), and T2DM (C2). The intervention was given through oral sonde for 28days. The variables analyzed were caecal-*cholesterol*-concentrations using a spectrophotometer and hs-CRP using the ELISA method. The statistical tests were used for the caecum-cholesterol-concentration and hs-CRP levels. The mean of caecum-cholesterol-concentration in the T3-group (83.68 ±1.93mg.100g^{-1}), was higher than T1 (77.99 ±1.70; p = 0.004) and C2-control (24.39 ±1.47; p = 0.0001). The mean of hs-CRP-level post-intervention in the T3-group (4.21 ±0.41 ng.mL^{-1}), was lower than C2 (17.15 ±0.85;p = 0.0001), T1 (6.59 ±0.27; p = 0.0001) and T2 (5.43 ±0.39; p = 0.004). There is a very strong inverse correlation between the concentration of cholesterol and hs-CRP (r = -0.979, p = 0.0001). The conclusion is *dadih*-fortified-vitamin-D_3 –intervention is better than its single intervention as an anti-inflammation which might relate to the increased caecum-cholesterol-concentration.

Keywords: T2DM; *dadih*; vitamin D_3; caecum-cholesterol; hs-CRP

INTRODUCTION
Diabetes Mellitus (DM) as a chronic disease is marked by the increase of the amount of blood glucose that resulted from the incapability of the pancreas to produce insulin or the incapability of the human's body to use existing insulin effectively **(WHO, 2016)**. DM prevalence in 2014 is 422 million and increase to 425 million in 2017. In 2017, The International Diabetic Federation (IDF) informed that Indonesia belongs to ten big countries with 10.3 million of DM patients of 20 to 79 years old **(IDF, 2015)**. Type 2 diabetes mellitus (T2DM) is the one commonly emerged. It reaches 90% of the total cases of DM **(IDF, 2015)**. T2DM is caused by a decrease in the body responds to insulin, known as insulin resistance. The inability of pancreatic-β-cells to maintain stable glucose balance causes the increase of plasma glucose levels (hyperglycemia) **(IDF, 2015; Kahn, Cooper and Del Prato, 2014)**.

T2DM-patients have significantly lower 25(OH)D-concentrations than healthy people **(Stivelman and Retnakaran, 2012)**. The hypovitaminosis D is one of the causes of glucose intolerance and the decrease of insulin secretion of T2DM either directly by activating vitamin D receptors (VDR) or indirectly by calcemic hormones and inflammation **(Shang and Sun, 2017)**.

Vitamin D acts as an anti-inflammatory and immunomodulatory of various diseases like obesity, cardiovascular, and diabetes **(Jafari et al., 2015)**. Vitamin D is also able to proliferate and stimulate T cells. It is also able to reduce pro-inflammatory mediators like C-Reactive Protein (CRP) **(Wu et al., 2014)**. Generally, fortification of vitamin D is done to dairy products such as yogurt

(Jafari et al., 2015). Supplementation of vitamin D and yogurt fortified with vitamin D has been shown to successfully improve the glycemic status of T2DM-patients **(Nakashima et al., 2016; Jafari et al., 2015)**. Yogurt fortified with vitamin D even reduces inflammation in postmenopausal women with T2DM **(Jafari et al., 2016)**. *Dadih* is an Indonesian traditional milk fermentation which has been shown to have a good impact on animal models of obesity **(Kusuma et al., 2015)**. The success of *dadih* fortified with vitamin D, however, has never been proven in T2DM.

Dadih is a product of spontaneous fermentation of buffalo milk done under a temperature of 28 – 32 °C for 24 – 48hours inside the bamboo tube **(Wijayanti, Thohari and Purwadi, 2016)**. Type of lactic acid bacteria (LAB) like *L. plantarum subsp. plantarum, Lactobacillus subsp. lactis, Lactococcus lactis subsp. cremoris, Lactobacillus brevis, Lactobacillus viridescens, Lactobacillus buccheri*are invented by isolating *dadih* of Lima Puluh Kota district **(Wirawati et al., 2018; Wirawati et al., 2019)**. *Dadih* gives several benefits such as antidiabetic, antiadipogenic, antiatherosclerotics, reducing the level of cholesterol. It can also improve human immunity **(Han et al., 2012; Wirawati et al., 2018)**.

Fermentation of buffalo's milk produces a peptide 4 – 20 kDa that resists free radicals affected by chronic inflammation and pancreatic beta-cell apoptosis **(Mustopa, Andrianto and Faridah, 2017)**. LAB can balance intestinal microbiota that can inhibit the production and secretion of lipopolysaccharides (LPS). The decrease of LPS content in the intestinal epithelium caused a decrease of proinflammatory cytokines **(Chuengsamarn et al., 2017; Park et al., 2013)**.

Dadih mechanism can reduce cholesterol through probiotics in *dadih* that assimilate cholesterol then cholesterol gets into the membrane of bacteria cell wall Absorption of cholesterol inside small intestine diminishes then cholesterol gets into caecum and at last, it is discarded through feces **(Nuraida, 2015; Kumar et al., 2012)**.

This study aimed to determine whether the intervention of *dadih*–fortified-vitamin-D_3 increased caecum-cholesterol-concentration and decreases hs-CRP levels in T2DM rats induced by a high-fat diet (HFD), streptozotocin (STZ) and nicotinamide (NA) injection.

Dosage of fortified *dadih*-vitamin-D_3 in this study was 36 IU.day^{-1} vitamin-D_3 on *dadih* 4 g.kg^{-1} weight body.day^{-1}. The intervention of *dadih*-fortified-vitamin-D_3 was carried out for 28days **(Kusuma et al., 2015; Jafari et al., 2015)**.

Scientific hypothesis

Dadih-fortified-vitamin-D_3 increases caecum-cholesterol concentration and decrease hs-CRP levels in T2DM Wistar rats.

MATERIAL AND METHODOLOGY

Dadih-fortified-vitamin-D_3 preparation and intervention

Dadih was made from buffalo milk from farms in the Gadut area, West Sumatra Province. Buffalo milk was pasteurized at temperature 72 °C for 15seconds. The milk was then cooled to 30 °C and added 900 IU of vitamin-D_3 then put into a bamboo tube and then it covered with banana leaves for two days of fermentation. The bamboo used was betung bamboo with a diameter of 4 – 5 cm, a length of 20 cm, and a volume of 100 mL **(Ranieri et al., 2009)**. *Dadih*-fortified-vitamin-D_3 was given orally using a feeding tube given to experimental Wistar rats. The dosage was 4g.kg^{-1} BW.d^{-1} **(Kusuma et al., 2015)**.

Research design and experimental animals

This research was a true-experimental study with a randomized pre-post test with a control group design. Animals used were male Wistar rats, aged 8 weeks, the weight was 150 – 200 g. They were acclimatized at the laboratory of the Center for Food and Nutrition Studies at Gajah Mada University, Yogyakarta. The rats were placed in individual stainless-steel cages at regulated a temperature of 21 °C. The rats were fed with 15 g.d^{-1} of the Comfeed II standard-diet during the non-HFD period and intervention. Rats received ad libitum water during the experiment. Animal laboratory guidelines used were from the Central Laboratory for Food and Nutrition Studies, Gajah Mada University (UGM), Yogyakarta.

Thirty rats were divided into five groups which were healthy controlled (C1), T2DM controlled (C2), and T2DM intervention (T1, T2, and T3). The T2DM-condition was induced by HFD, STZ, and NA. After a week of acclimatization, rats were conditioned in T2DM with oral HFD 15 g.d^{-1} for 14 days then injected intraperitoneally with STZ 45 mg.kg^{-1} rat BW and NA 110 mg.kg^{-1} rat BW. T2DM condition was indicated by fasting blood glucose serum >250 mg.dL^{-1} **(Gheibi, Kashfi and Ghasemi, 2017)**. The T3-group was then treated with *dadih*–fortified-vitamin-D_3 in doses of 4 g.200g^{-1} BW.d^{-1} every day for the intervention of 28 days, while T1 and T2-groups were given vitamin-D_3 36 IU.d^{-1} and *dadih* 4 g.200g^{-1} BW.d^{-1}, respectively **(Kusuma et al., 2015; Jafari et al., 2015)**. This study was approved by the Health Research Ethics Diponegoro University Semarang through ethical clearance No. 140/EC/H/KEPK/ FK-UNDIP/XI/2019.

Determination of biochemical markers

The blood sample was taken first and at the end of the intervention, fasting blood glucose was taken through the retroorbital plexus. Blood glucose determination using GOD-PAP (BIOLABO SA). Determination of blood glucose after enzymatic oxidation by glucose oxidase. Blood samples were collected in a centrifugation tube and centrifuged 4000 rpm in 15 minutes (HerauesSepatech, Biofuge 15).

Caecum-cholesterol-concentration determination using the Lieberman Burchand method and analyzed by Spectrophotometer (Optima SP. 300). The sample's absorbance was further read by a spectrophotometer at 680 nm **(Plummer, 1971)**.

Figure 1 *Dadih* fermented.

Figure 2 *Dadih*-fortified-vitamin-D_3.

hs-CRP levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Fine Test, Wuhan Fine Biotech, China) according to manufacturer instructions **(Fine Test, 2019)**. Briefly, Reagen adds samples 100 µL of the properly diluted sample into test sample wells. The plate sealed with a cover and incubated at 37 °C for 90 minutes. After incubation, the plate was rinsed 3 times with wash buffer and let the Wash Buffer stay in the wells for 1 – 2 minutes each time. 100 µL of Biotin-labelled antibody working solution into the above wells (standard, test sample, and blank wells), sealed with a cover, and incubate at 37 °C for 60 minutes. After incubation, remove the cover and wash the plate 3times with Wash Buffer. 100 µL of SABC (Streptavidin Conjugate) Working Solution added into each well, sealed with a cover the plate, and incubated at 37 °C for 30 minutes. After incubation Remove the cover and wash the plate 5times with Wash Buffer. 90 µL TMB Substrate added into each well, sealed with a cover the plate, and incubated at 37 °C for 10 – 20 minutes in dark. After incubation, 50 µL Stop Solution added into each well. The color will turn yellow immediately. The absorbance of samples in well plates was read by ELISA Reader at 450 nm (Zenic).

Statistical analysis

Data were analyzed using version 21 of the statistical package for the Social Sciences (SPSS). The results were expressed as mean ±*SD* (for normally distributed data) otherwise it expressed as median (min-max). Statistical difference was analyzed by using One-Way Analysis of Variance (ANOVA) followed by Post Hoc Bonferroni for normally distributed data, otherwise, the Kruskal-Wallis test followed by Mann Whitney test was used. The Differences before and after intervention were analyzed using a Paired *t*-test for normally distributed data, otherwise the Wilcoxon test. Pearson's correlative test was used to analyze the correlation between variables, and statistical analyses were performed by a computer. The differences and correlations were considered significant at $p < 0.05$ and 95% confidence intervals (CI). The strength of correlations was determined by the r value.

RESULTS AND DISCUSSION

The data processed in this study was obtained from 29 Wistar rats, which are divided into each group consisting of 6 rats. The use of *dadih* in this study referred to the kefir study. Kefir intervention. the study of T2DM patients indicates that LAB in kefir contributes to the regeneration of pancreatic beta cells and then it increases the ability to produce insulin. Further, insulin facilitates the glucose absorption into adipose and liver that glycogenesis and lipogenesis occur. This process might contribute to increasing the weight of T2DM receiving kefir **(Ostadrahimi et al., 2015)**.

The blood glucose levels are collected in all intervention groups (T1, T2, and T3). Before the intervention, all T2DM-rats showed significantly higher blood glucose than C1-group (Post Hoc Bonferroni test T1, $p = 0.0001$; T2, $p = 0.0001$; T3, $p = 0.0001$, Table 1). The blood glucose before the intervention also showed that there was no difference between the intervention group ($p < 0.05$). The blood glucose levels decreased significantly after treatment in all treatment groups (paired *t*-test; $p<0.05$). The post-intervention of blood glucose showed that all treatment group was significantly lower than C2-group (Post Hoc Bonferroni test T1, $p = 0.0001$; T2, $p = 0.0001$; T3, $p = 0.0001$). Additionally, those of treatment groups remained higher than C1-group (Post Hoc Bonferroni test T1, $p = 0.0001$; T2, $p = 0.0001$; T3, $p = 0.003$). This indicated that all interventions used improved glycemic status, although it had not reached a healthy yet at a group level. The post-intervention blood glucose level in the T3 group had the lowest level among intervention-groups and significantly different than the T1 group. Intervention with *dadih*-fortified-vitamin-D_3 was proved that the glucose level was able to decrease better than the single intervention (vitamin D_3).

The pre-post intervention change (Δ) of blood glucose levels was significantly different among the five groups (Kruskal-Wallis test, $p = 0.0001$). Results of the Mann Whitney test showed the significant differences in blood glucose levels ($p < 0.05$) between the intervention groups (T1, T2, T3) compared to the C1-group. The negative Δ blood glucose level of T3 was bigger than T1 and T2, and this was significantly different. This gave an additional finding that *dadih*-fortified-vitamin-D_3-intervention was not only better than the single intervention (vitamin-D_3) but also *dadih*.

The result of the One-way ANOVA test showed that there was a significant difference in caecum-cholesterol-concentration among groups in this study ($p = 0.0001$). Caecum-cholesterol-concentrations of T1, T2, and T3-groups was significantly higher than C2-groups (Table 2; Post Hoc Bonferroni test; $p = 0.0001$ in each group). These indicated that vitamin-D_3, *dadih,* and *dadih*-fortified-vitamin-D_3 the treatments contribute to the increased of caecum-cholesterol- concentrations of T2DM-rats. Interestingly, those of T2 and T3-groups were significantly higher than the T1-group ($p = 0.0001$ and $p = 0.004$). Also, those of the T2-group were significantly higher than the T3-group ($p = 0.003$). Therefore, *dadih*-intervention might have a predominant-effect in increasing caecum-cholesterol-concentration in T2DM-rats. Probiotics contribute to reducing cholesterol absorption in the intestine. This is based on the yogurt study. Probiotics assimilate cholesterol and get the cholesterol into the membrane of probiotic cells. As a result, the cholesterol absorption in the intestine reduces and the cholesterol will get into the cecum to be disposed of with feces **(Ejtahed et al., 2011; Nuraida, 2015)**. Probiotics can reduce cholesterol concentrations in vitro through the conversion of cholesterol into coprostanol by cholesterol reductase. This condition leads to the cholesterol excretion in feces increases, while cholesterol in the plasma decreases **(Lye, Rusul and Liong, 2010)**. Vitamin D mechanisms used in increasing caecum-cholesterol-level have not been elucidated. However, vitamin D involves the decline of blood cholesterol levels. Vitamin D inhibits HMG-CoA reductase, an enzyme function in endogenous cholesterol biosynthesis and it can also de-conjugate bile acids in the intestine, and subsequently, it reduces cholesterol concentration **(Kane et al., 2013; Ostadrahimi et al., 2015)**. The caecum-cholesterol-concentration in those treatment-groups (T1, T2, and T3) at the post-intervention was significantly lower than healthy C1-group ($p = 0.0001$, $p = 0.006$, and $p = 0.0001$). This suggests that improving interventions remain open for the benefit of T2DM-individuals.

All of the treatment-groups had lower hs-CRP levels at the post-intervention, and these differences were significant (Table 1; Wilcoxon test, $p = 0.028$ in each group). All of the treatment-groups had lower hs-CRP levels than C2-group at the post-intervention. The negative delta (Δ) of hs-CRP levels was bigger in those receiving treatments than C2-group (Mann Whitney test; T1, $p = 0.006$; T2, $p = 0.004$ and T3, $p = 0.004$). All of these showed that all interventions used in this study were associated with the reduction of blood hs-CRP levels. The post-intervention of hs-CRP levels in the T3-group (Mean ±*SD*; 4.21 ±0.41 ng.mL^{-1}), was lower than C2 (17.15 ±0.85; $p = 0.0001$), T1 (6.59 ±0.27; $p = 0.0001$) and T2 (5.43 ±0.39; $p = 0.004$). Thus, *dadih*-fortified-vitamin-D_3 intervention might have a better effect than its single intervention to decrease blood-hs-CRP levels in T2DM-rats. *Dadih*contains LAB and the previous studies show that LAB in fermented food involves mechanisms to reduce blood-CRP-levels. LAB induces the increase of production of several anti-inflammatory cytokines, and the decrease of COX-2 expression, an enzyme that catalyzes the production of prostaglandins from arachidonic acid, in which it stimulates cell proliferation and inflammatory processes **(SaeidiFard, Djafarian and Shab-Bidar, 2020)**. LAB balances the gut microbiota therefore, it inhibits the production and secretion of LPS. Decreased of LPS in the intestinal epithelium causes a decrease in proinflammatory cytokines **(Wirawati et al., 2019)**. Probiotics from *dadih* can also produce SCFA in the colon, while this SCFA can reduce the synthesis of CRP enzymes in the liver **(Asemi et al., 2013)**.

Vitamin D can provide a protective effect by reducing sensitivity to the circulating-CRP **(Zakharova et al., 2019).** Vitamin D does not only decrease CRP but also decreases proinflammatory cytokines including TNF-α, IL-6, IL-1β **(Mirhosseini et al., 2017)**. Vitamin D weakens the expression of proinflammatory cytokines involved in insulin resistance and regulates NF-Kb activities.

Table 1 BodyWeight, BloodGlucoseandhs-CRP Level.

Groups	C1	C2	T1	T2	T3	p^1
Marker						
BodyWeight of Intervention (g)						
Pre	182.5	165.5	165.00	174.50	160.50	0.004
Post	(172.0-211.0)^{b,c,d,e}	(150.0-182.0)^{a}	(145.00-173.00)^{a,d}	(150.00-180.00)^{a,c,e}	(148.00-170.00)^{a,d}	
Δ	220.50 ±33.02^{b}	108.50 ±14.85^{a,c,d,e}	177.80 ±16.78^{b}	209.67 ±23.80^{b}	182.67 ±26.03^{b}	0.0001
	33.0	-55.0	11.00	37.50	14.50	0.005
p	(4.00-67.00)^{b}	(-69.00-(-47.00))^{a,c,d,e}	(-3.00-37.00)^{b}	(-7.00-71.00)^{b}	(6.00-51.00)^{b}	
	0.027	0.027	0.080	0.046	0.026	
Blood Glucose Level (mg.dL^{-1})						
Pre	70.67 ±1.52^{b,c,d,e}	288.04 ±4.46^{a}	284.21 ±2.66^{a,}	286.14 ±2.02^{a,}	289.52 ±4.02^{a}	0.0001
Post	72.96 ±0.96^{b,c,d,e}	290.87 ±4.72^{a,c,d,e}	101.83 ±2.32^{a,b,d,e}	83.80 ±3.72^{a,b,c}	80.32 ±1.17^{a,b,c}	0.0001
Δ	2.36	3.03	-180.64	-202.57	-207.83	0.0001
	(1.21-2.96)^{c,d,e}	(1.99-3.44)^{c,d,e}	(-189.25-(-180.35))^{a,b,d,e}	(-209.11-(-194.36)^{a,b,c,e}	(-215.49-(-204.67)^{a,b,c,d}	
p	0.0001	0.0001	0.0001	0.0001	0.0001	
***hs-CRP Level* (pg.mL^{-1})**						
Pre	3.05	16.36	16.42	16.88	16.91	0.005
Post	(2.88-3.60)^{b,c,d,e}	(16.23-18.24)^{a}	(16.33-17.56)^{a}	(16.07-18.43)^{a}	(15.55-18.61)^{a}	
Δ	3.24 ±0.28^{b,c,d,e}	17.15 ±0.85^{a,c,d,e}	6.59 ±0.27^{a,b,d,e}	5.43 ±0.39^{a,b,c,e}	4.21 ±0.41^{a,b,c,d}	0.0001
	0.09	0.26	-10.15	-11.45	-13.04	0.0001
p	(0.04-0.16)^{b,c,d,e}	(0.19-1.64)^{a,c,d,e}	(-10.99-(-9.50))^{a,b,e}	(-12.47-(-10.95))^{a,b}	(-14.38-(-10.76))^{a,b,c}	
	0.028	0.028	0.043	0.028	0.028	

Table 2 Caecum-Cholesterol-Concentration Test.

Rat Group	Caecum Cholesterol Concentration (Mean ±*SD*) (mg.100g^{-1})
C1	94.57 ±2.67 b,c,d,e
C2	24.39 ±1.47 a,c,d,e
T1	77.99 ±1.70 a,b,d,e
T2	89.34 ±3.20 a,b,c,e
T3	83.68 ±1.93 a,b,c,d

Note: Alphabetical superscripts showed a significance level of ^{a}: p <0.05 compared as C1; ^{b}: p <0.05 as compared to C2; ^{c}: p <0.05 as compared to T1; ^{d}: p <0.05 as compared to T2; ^{e}: p <0.05 as compared to T3.

Table 3 Correlation studies of caecum-cholesterol-concentration and hs-CRP levels with other biochemical markers.

	hs-CRP levels		Blood Glucose Level	
	r	***p***	**r**	***p***
Caecum-cholesterol-concentration	-0.979	0.0001	-0.988	0.0001
Blood Glucose Level	0.988	0.0001		

Vitamin D can also improve the composition and function of intestinal microbiota, reduce pathogenic bacteria, and increase bacterial diversity. Another study in Rotterdam shows that Vitamin D deficiency is prevalent in overweight and obese children and adolescents **(Zakharova et al., 2019)**. Additionally, the higher vitamin D levels are associated with the lower of CRP-levels in T2DM-patients **(Liefaard et al., 2015)**. However, the study has not proved a causal relationship between vitamin D and CRP-level. The recent study used interventions of vitamin D_3 and *dadih*-fortified-vitamin-D_3 was strengthening the role of vitamin D_3 to lessen CRP-level.

The Spearman test on all data from T2DM rats at the end of the study showed that a very strong positive correlation was observed between hs-CRP levels and blood glucose levels (Table 3), while a very strong negative correlation was found between caecum-cholesterol-concentration either with hs-CRP levels or blood glucose levels.

A very strong negative correlations was observed between caecum-cholesterol-concentration and hs-CRP-levels (r = -0.979, p = 0.0001, Table 3). Caecum cholesterol is the cholesterol that is secreted by the body and will be excreted with fecal. An increased concentration of cholesterol excreted from the body will reduce the bile acids returning to the hepatic cycle, and subsequently, the cholesterol in blood circulation will be consumed for synthesizing new bile acids **(Kumar et al., 2012; Kusuma et al., 2015)**. The very strong inverse relation between caecum cholesterol and hs-CRP levels may be mediated by the decline circulating cholesterol levels which relate to the decline in inflammation.

The increase of fatty acids or triglycerides in adipose tissue will cause enlargement in adipose tissue and activate the HF-1 gene. This gene increases the expression of Jun kinase and IkB kinase and triggers phosphorylation of IkB and activates NFkB. It will trigger the expression of proinflammatory cytokines such as TNF-α and IL-6. This

increase of cytokines will trigger the liver to secrete CRP **(Qatanani and Lazar, 2007)**. Plasma CRP levels are significantly correlated with high triglycerides and low HDL. CRP is synthesized in adipose tissue, the amount will increase in a state of obesity which will ultimately cause insulin resistance and diabetes **(Devaraj, Singh and Jialal, 2009)**.

A very strong negative correlation was observed between caecum-cholesterol-concentration and blood-glucose-levels (r = -0.988, p = 0.0001). Glucose levels increase in groups that had low caecum-cholesterol-concentration. T2DM is associated with changes in the composition of intestinal microflora, namely an increase in the number of gram-negative bacteria and a decreased proportion of Firmicutes. The individuals with T2DM, therefore, have lipid metabolism abnormalities **(Ejtahed et al., 2011)**.

Lipid metabolic disorders caused by insulin resistance affect Hydroxymethyl Glutarate Coenzyme-A (HMG-CoA) reductase and lipid metabolic pathways. Several studies have shown that insulin can affect the production of apolipoprotein in the liver and regulate the activity of lipase which causes dyslipidemia in diabetes mellitus **(Ozder, 2014)**. Excessive accumulation of fat in adipose tissue causes macrophage infiltration and increases the production of proinflammatory cytokines which contributes to the development of atherosclerosis **(Pei et al., 2017)**.

A very strong positive correlation was found between hs-CRP-levels and blood-glucose-levels (r = 0.988, p = 0.0001). This correlation showed that increase glucose levels relate to enhanced hs-CRP-levels. Hyperglycemia causes damage to all body tissues and affects the chronic inflammatory response such as hs-CRP. Also, hyperglycemia in cells causes damage to the mitochondria. It will increase Reactive Oxygen Species (ROS) so that the amount of free radicals increase in the body. Furthermore, hyperglycemia also increases the synthesis of diacyl glycerides (DAG) which causes activation of Protein Kinase C (PKC) and ultimately changes the expression of various genes that will damage blood vessels. Increased activity of PKC leads to activation of NF-kB and it induces various pro-inflammatory cytokines such as TNF-α and IL-6 which trigger the liver to produce CRP **(Nowotny et al., 2015)**.

CONCLUSION

Dadih-fortified-vitamin-D_3-supplementation has a better effect than either *dadih* or vitamin-D_3 in decreasing blood-glucose and hs-CRP levels. *Dadih*-fortified-vitamin-D_3supplementation has better effect than vitamin-D_3 in increasing caecum-cholesterol-concentration T2DM-rat. Both hs-CRP andglucose levels havea very strong inverse relationship withthe caecum-cholesterol-concentrations.

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THE OCCURRENCE OF ELEVEN ELEMENTS IN DAIRY COW´S MILK, FEED, AND SOIL FROM THREE DIFFERENT REGIONS OF SLOVAKIA

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ABSTRACT

The objective of this study was to measure the concentrations of eleven essential, potentially toxic and toxic elements (arsenic – As, calcium – Ca, cadmium – Cd, copper – Cu, iron – Fe, mercury – Hg, magnesium – Mg, nickel – Ni, lead – Pb, selenium – Se, zinc- Zn) in raw cow's milk (spring, summer, and autumn season), feed (spring and autumn season) and soil (spring season) from three different environments by routine methods in the certified testing laboratory. The samples were collected in the undisturbed region around Novoť, the moderately disturbed region around Tulčík, and the strongly disturbed region around Čečejovce. The concentrations of all toxic elements (As, Cd, Hg, Ni, Pb) and two essential elements (Cu, Se) in milk were under the limits of quantification (LOQ) from all investigated areas and during all seasons. Concentrations of other elements in milk from the undisturbed and disturbed areas were significantly different, generally with the highest levels in summer. In soil samples, the significantly highest concentrations of Ca, Cu, Ni were found in a strongly disturbed area, Mg and As in moderately disturbed area, and Fe, Se, Zn, Hg, and Pb in an undisturbed area. Cadmium was under the LOQ. In feed, the concentrations of essential elements, except of Se, were higher in the autumn. The significantly highest concentration of As, Ni were recorded in a moderately disturbed area and Pb in the undisturbed area in both seasons. Cadmium and Hg were under the LOQ. Despite the higher level of some elements in soil (Fe, Mg, Ca) from all regions, there were not elevated concentrations of any element in feed or milk. The concentrations of all toxic elements in milk were under the permitted limits. Thus, the milk from all investigated areas was not contaminated with the elements posing a health risk for consumers and it is considered safe for human consumption.

Keywords: essential element; toxic metal; cow milk; feed; soil

INTRODUCTION

Milk is a well-known source of many compounds with a beneficial role in the human organism. It contains essential elements, vitamins, proteins, and other compounds important mainly for the children's health. However, except for these important compounds, it may contain also toxic or potentially toxic elements. **Hermansen et al. (2005)** analysed 45 trace elements and 6 macro elements (Ca, potassium – K, Mg, sodium – Na, phosphorus – P, sulphur – S) in cow milk. Authors found the differences between the organically produced milk and conventionally produced milk with elevated concentration of molybdenum (Mo) in organic milk, whereas the concentrations of barium (Ba), europium (Eu), manganese (Mn), and zinc (Zn) were significantly reduced compared with conventional milk. Differences in element concentrations based on animal species and regions reported **Zhou et al. (2017)**. Authors also found that the concentrations of elements in water and feed might contribute to those in milk. Toxic and potentially toxic elements in raw milk were associated with those in feed and drinking water. Results of meta-analysis made by **Zwierzchowski and Ametaj (2018)** show that concentrations of Pb were above the minimum-risk level (MRL) in the milk samples from Brazil, Croatia, Egypt, Mexico, Nigeria, Palestine, Romania, Serbia, and Turkey. Moreover, organic dairy farms are characterized by lower concentrations of toxic heavy metals in whole raw milk compared with those from the conventional production system.

A large proportion of the total amounts of the elements contains casein fraction in cow's milk but not in humans **(Fransson and Lönnerdal, 1983)**. Lead and As were found in the cow's milk from areas irrigated with wastewater in Mexico with Pb concentrations above the maximum limit as set by Codex Alimentarius and the European Commission standards **(Castro-González et al., 2018)**. **Castro-González et al. (2019)** warn that chronic heavy metal consumption in contaminated cow's milk can pose a serious

health risk for girls and children. The heavy metals in the milk had the following order Zn >As >Pb >Cr >Cu >Ni. Lead exceeded the Codex limits. Many of the metals occurring in the milk can cause cancer in humans. As, Cd, chromium (Cr), Pb, and Hg are considered systemic toxicants and are also classified as human carcinogens **(Tchounwou et al., 2012)**. Epidemiological studies have shown that As exposure is associated with a variety of human cancer of the skin, lungs, bladder, liver. Exposure occurs primarily via drinking water, but dietary exposure can also be substantial **(Zhou and Xi, 2018)**. Environmental quality and human activities (soil, water, river, industry, mining, and smelting) play a key role in the distribution of toxic metals in raw milk and contribute to Pb, As, and Cd contamination in animals and transfer to milk **(Kazi et al., 2009; Zhou et al., 2019a)**. In bovine milk, Hg is associated with two protein fractions, caseins, and beta-lactoglobulin **(Mata, Sanchez and Calvo, 1997)**. Mercury in cow milk samples in concentration 3.1 $ng.g^{-1}$ found **Najarnezhad and Akbarabadi (2013)**. Nickel was also found in cow milk and high concentrations of Ni in traditional farms compared to industrial farms could be attributed to the location of this industry in a rural area **(Arianejad et al., 2015)**.

Milk may be an important source of essential elements. The iron content of cow's milk is about 0.5 $mg.L^{-1}$ and is thus comparable to that of human milk **(Ziegler, 2011)**. However, cow's milk and Ca inhibit nonheme Fe absorption **(Domellöf et al., 2014)**. Copper is found in lower concentrations in milk. However, the concentrations of Cu in the meat and cow milk samples were higher than the maximum allowable concentration (MAC) of Cu in foods in Bangladesh **(Shaheen et al., 2016)**. Even higher Cu concentrations were found in Croatia **(Bilandžić et al., 2011)**. Milk can significantly contribute to the dietary Se intake in human. Higher milk Se concentrations have been measured in the Northern Ireland, but its content in milk is affected by geographical location **(O'Kane et al., 2018)**. Effect of parity, stage of lactation and breed on mineral composition of cow milk reported **Manuelian et al. (2018)**. Milk of primiparous cows had greater Ca, Mg, K, and P contents than milk of multiparous cows. Holstein-Friesian produced the lowest concentrations of Ca, Mg, and P content. Jersey yielded milk with the greatest Ca and Mg content.

Due to the importance of milk consumption and its content of essential and toxic elements mainly for children's health, the aim of this work was to analyse and compare the occurrence of 11 elements in raw cow's milk, feed and soil samples from different areas of Slovakia based on the environmental regional classification.

Scientific hypothesis

The concentration of toxic and essential elements in cow's milk is affected by the environmental quality.

MATERIAL AND METHODOLOGY

Sample collection

The cow's milk, feed and soil samples were collected in three different areas of Slovakia based on the environmental regional classification **(MESR and SEA, 2018)** (Figure 1). The location around Novoť is considered as a region with an undisturbed environment, the second analysed area of Tulčík is considered as a region with a moderately disturbed environment, and the third area considered as a region with a strongly disturbed environment is located around Čečejovce.

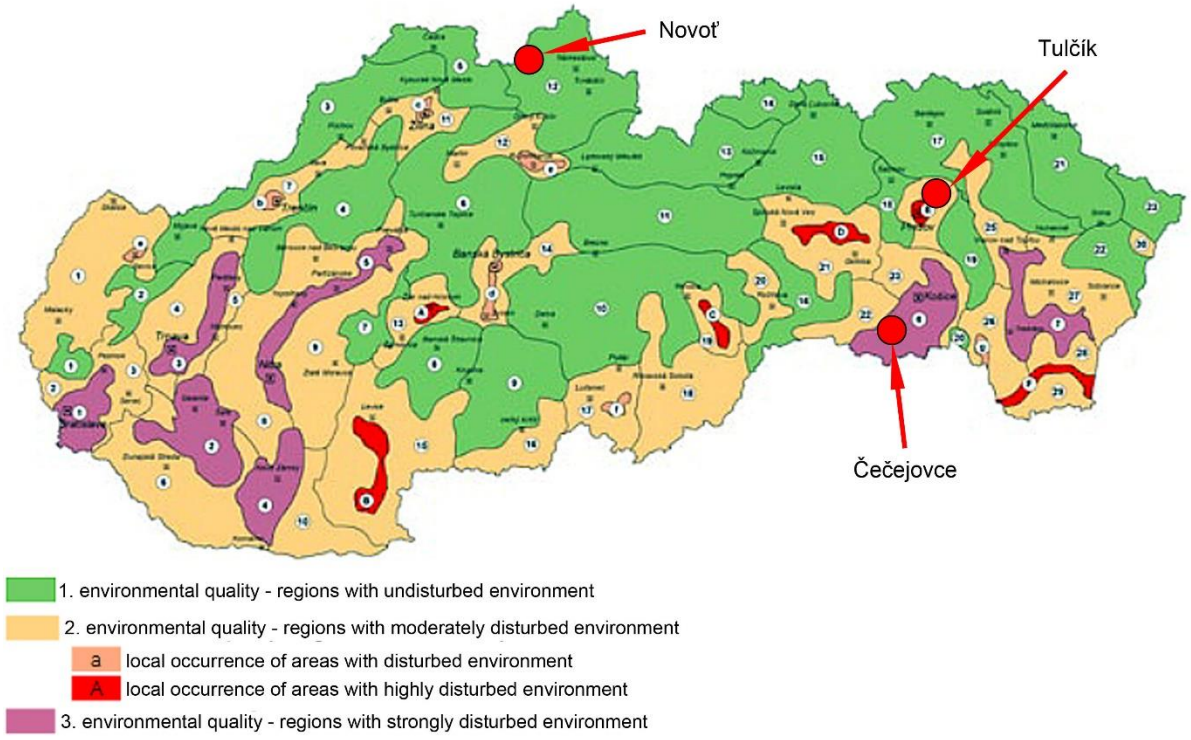

Figure 1 Environmental regional classification of Slovakia **(MESR and SEA, 2018)**. Note: Three investigated regions (Novoť, Tulčík, Čečejovce) are marked.

The tank milk samples were collected immediately after the cows were milked, in the morning and in the afternoon by an automatic milking system. Samples were collected three times a year; in spring (April), in summer (July), and in autumn (September). 500 mL of milk were collected directly from the milk tank during the two days (two times from morning milking and two times from afternoon milking). Five samples of milk were collected from each milking, in total 60 samples of pool milk (20 samples each season) from each location. The total number of the dairy cows were as follows: Novoť area (220 cows; crossbreeds: Slovak spotted breed × Red Holstein breed), Tulčík area (450 cows; Slovak spotted breed), and Čečejovce area (340 cows; Black Holstein breed). Average milk samples from these cows were obtained from milk tanks immediately after the end of milking. Samples were kept in PET bottles at -18 °C until analysis.

Ten feed samples (5 in April and 5 in September) of total mixed ration (TMR) were collected from each observed location. The feed was made at all farms from local components from these farms. Samples were stored in plastic bags at -18 °C until analysis.

Five soil samples were collected from different places at each farm during the spring season (April). Samples were stored in plastic bags at -18 °C until analysis.

Sample analysis

Milk samples for Ca, Fe, Mg, Cd, Ni, Pb, Cu, Zn analyses were mineralized by microwave decomposition with HNO_3 and H_2O_2 (microwave oven MARS 6 240/50). Milk samples for As analysis were prepared by dry mineralization with oxidation mixture (oxygen, oxides of nitrogen, ozone), heated at 300 – 400 °C. The ash was re-diluted in HCl solution. Milk samples for Se analysis were mineralized by microwave decomposition with HNO_3 and H_2O_2 (microwave oven MARS 6 240/50) and after removal of nitrous gases, cooling, and addition of HCl solution, Se^{6+} was reduced to Se^{4+} by heating at 90 °C.

Feed samples were prepared by dry mineralization in owen at 475 °C (Ca, Fe, Mg, Cu, Zn) or 580 °C (Cd, Ni, Pb) and the ash was diluted in HCl solution. Feed samples for As and Se analyses were prepared by magnesium nitrate and oxide suspension and mineralized with HNO_3 at 470 °C. The ash was diluted in the HCl solution. Se^{6+} was reduced to Se^{4+} by heating at 90 °C.

Soil samples for As, Se, Ca, Fe, Mg, Cd, Ni, Cu, Pb, Zn were extracted with aqua regia and cooled for 2 hours.

Arsenic and Se in milk, feed, and soil were analysed using the hydride generation atomic absorption spectroscopy (HG-AAS) method with SpectrAA-220 FS (The Netherlands). Calcium, Fe, and Mg in milk, feed, and soil samples were detected using the inductively coupled plasma-atomic emission spectrometry (ICP-AES, Varian 720-ES, USA). Cadmium, Pb, and Ni in milk and feed were analysed using the electrothermal atomization atomic absorption spectrometry (ETA-AAS, Agilent DUO AA 240Z/240FS, USA). Zinc and Cu in milk and feed and Cd, Ni, Cu, Pb, Zn in soil were analysed using the flame atomic absorption spectrometry (F-AAS, DUO AA 240Z/240FS, USA). Mercury in milk, feed, and soil samples was analysed using the Advanced Mercury Analyzer and atomic absorption spectrometry (AMA-AAS, AMA254, Altec, Czech Republic) without the need for chemical preparation of the sample. All analyses were conducted in certified testing laboratory Eurofins/Bel Novamann (Nové Zámky, Slovak Republic).

Quality assurance

For the validation of the analytical methods the limits of detection (LOD) and LOQ were evaluated (Table 1). LOD in digest was calculated as three times the standard deviation of the sample blank relative to the slope of the analytical curve. LOQ was calculated as 10 times the standard deviation of the sample blank relative to the slope of the analytical curve. LOD and LOQ were calculated separately for soil, feed, and for food in general. Based on the obtained LOD limits, LOQ limits according to the needs of **Commission Regulation (EC) no. 1881/2006** were obtained. LOQ limits were recalculated from $mg.L^{-1}$ (LOD) to $mg.kg^{-1}$ based on the sample weight and the final volume of the digest. The quality control (QC) during measurement was ensured by a parallel analysis of at least one sample and the method calibration was controlled before every measurement by the control sample from the calibration solution of certified reference material (CRM). The solution was prepared by diluting a standard solution (Ultra Scientific, USA) with a certified value of 1000 $mg.L^{-1}$ (multi-element solution).

Table 1 LOD and LOQ values for essential and toxic elements analysed.

Element	LOD ($mg.kg^{-1}$)			LOQ ($mg.kg^{-1}$)		
	milk	feed	soil	milk	feed	soil
Ca	2.0	2.0	2.3	6.0	6.0	7.0
Cu	0.017	0.17	0.3	0.05	0.50	1.0
Fe	0.17	0.17	3.0	0.50	0.50	10.0
Mg	0.33	0.33	0.3	1.0	1.0	1.0
Se	0.0067	0.017	0.06	0.03	0.050	0.20
Zn	0.17	0.17	2.0	0.50	0.50	6.0
As	0.01	0.017	0.06	0.03	0.050	0.20
Cd	0.0013	0.0067	0.13	0.0040	0.10	0.40
Hg	0.00067	0.0033	0.003	0.002	0.010	0.010
Ni	0.03	0.033	0.8	0.10	0.10	2.5
Pb	1.0	0.033	1.0	0.01	0.10	3.0

A standard from a different supplier was used to prepare the QC sample, and then the standard was used to prepare the calibration solutions.

The accuracy of the method, CRM, skimmed milk powder (ERM-BD151) was analysed for the determination of elements in milk, CRM, poultry feed-proximates and elements (LGC7173) and pig feed (BCR-709) for elements in feed and quality control material (CQM) river sediment (Metranal TM 1) for elements in soil samples.

Statistical analysis

Statistical analysis of the data was performed using SAS 9.2 (SAS Institute Inc., USA). Differences in concentrations of the analyzed elements in feed, soil, and cow's milk between seasons and three investigated areas were compared by the ANOVA and Student's t-test. All data were expressed as mean, standard deviation, and coefficient of variation. A probability level of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Except of Cu and Se, all analysed essential elements were present in all milk samples (Table 2). The significantly highest Ca concentration was found in the undisturbed area of Novoť in spring and summer in comparison to the other two areas. Significantly lowest Ca concentration in spring was found in milk from the Tulčík area ($p < 0.01$), in summer and autumn in the Čečejovce area ($p < 0.001$ and $p < 0.05$, respectively). The effect of season on Ca concentration in cow milk is contradictory in the literature. **Lin et al. (2017)** reported that season had no significant effect on total Ca in milk. Also, **Chassaing et al. (2016)** did not confirm the seasonal changes of mineral contents in cow milk. The concentrations of Ca in cow milk vary and ranged from 1203.5 to 1316.1 mg.kg^{-1} **(Toffanin et al., 2015)**, 1043 – 1283 mg.kg^{-1} **(Gaucheron, 2005)**, 1409.86 – 2156.45 mg.kg^{-1} **(Pilarczyk et al., 2013)** up to 3789.7 mg.L^{-1} **(Capcarova et al., 2019)**, and in our analyses, they ranged from 981 mg.kg^{-1} (Tulčík in spring) to 1930 mg.kg^{-1} (Novoť in summer). Moreover, **Toffanin et al. (2015)** found that Ca milk content decreased between March and May. Similar trends in Ca milk concentration were found in our analyses and are supported by findings of **Hurtaud et al. (2014)** and **Poulsen et al. (2015)**. **Boudon et al. (2016)** suggest that long and sunny days could explain part of the seasonal decrease in milk Ca content in summer.

Significantly lowest Fe concentration in milk was found in the Čečejovce area in spring ($p < 0.01$) and in autumn ($p < 0.05$). Significantly lowest Mg concentration was found in Tulčík in spring and summer ($p < 0.001$) and in Čečejovce in autumn ($p < 0.01$). The highest Mg concentration was found in Tulčík during the autumn season ($p < 0.001$) in comparison to the undisturbed area of Novoť, where the Mg concentration was lowest in autumn. The same and lowest Zn concentrations in milk were recorded around Tulčík and Čečejovce with a significant decrease in spring ($p < 0.001$). In summer, the lowest Zn concentration was found in the Tulčík area ($p < 0.01$) and in autumn in the Čečejovce area ($p < 0.01$). In the previous study, **Pšenková et al. (2020)** detected in the strongly disturbed environment of the Čečejovce area in 2016 only Ca, Mg, and Zn in the cow milk. In our study, the Ca, Mg, and Zn concentrations were about 2 – 3 times higher. High level of variability in the Ca, Cu, Fe, Na, Ni, and Zn contents in cow milk found **Capcarova et al. (2019)** in Slovakia (SK) and Czech Republic (CZ). The concentrations of observed elements in SK and CZ milk built an order of increasing concentrations: Ni <Cu <Fe <Zn <Mg <Na <K<Ca. The same trend was observed in our experiments, no matter the season and area. A similar concentration of Zn in cow milk was found by **Wiking, Larsen and Sehested (2008)**, **van Hulzen et al. (2009)**, **Stocco et al. (2019)**. The Mg concentrations in milk were in the normal range 97 – 146 mg.kg^{-1} as described **Gaucheron (2005)** with slightly higher levels in summer (154 – 164.2 mg.kg^{-1}) in all areas. Similar results recorded **Pilarczyk et al. (2013)** at an organic farm in Poland. The concentrations of essential elements in milk from all areas were in the normal range meaning there was no obvious contamination. A different situation was recorded in Egypt. The analyses of Fe (16.38 mg.kg^{-1}), Zn (10.75 mg.kg^{-1}), and Cu (2.83 mg.kg^{-1}) showed that most of the cow milk samples from the different sites contained all the studied metals with a concentration higher than those recommended for milk by the international dairy federation standard and Codex **(Malhat et al., 2012)**. The element with the highest frequency of occurrence in cow milk in Turkey was Zn, followed by Cr = As >Al >Se >Fe >Ni >Cu >Pb = Cd, in decreasing order. The lowest concentration among the essential elements was seen in Cu. Al and As were very often found but Pb and Cd were not found in the milk samples **(Totan and Filazi, 2020)**. In our samples, the lowest concentration of the essential elements was found in Fe in the range of 0.404 – 0.626 mg.kg^{-1} aside from the season and area. On the contrary, average concentrations of Fe in all samples of milk analyzed in Palestine were the highest (2.01 – 3.86 mg.kg^{-1}) **(Abdulkhaliq et al., 2012)**. In our study, the concentrations of toxic and potentially toxic elements (As, Cd, Ni, Hg, Pb) in cow milk from all analyzed areas and in any season were under the LOQ (Table 1). It is important information due to the toxic nature of these elements. The milk from the investigated areas was not contaminated with the elements posing a health risk for consumers and it is considered safe for human consumption. In contrast, **Pilarczyk et al. (2013)** found Pb content in the milk of cows of two breeds two times higher than the permissible concentration of 0.02 mg.kg^{-1} in the raw milk given by the standards of the **Commission Regulation (EC) no. 1881/2006**. **González-Montaña et al. (2019)** also found some of the metals in milk (aluminium – Al, As, Mo) in the area with various anthropogenic activities (industrial, mining, traffic density). **Datta et al. (2012)** warned that consumption of milk from the contaminated areas might have produced arsenicosis and may be considered as an alternative source of arsenic contamination. Milk samples collected from the nonindustrial region of Turkey in the summer had higher Cr, Mn, and Zn concentrations than the polluted region. However, industrial activities and seasonal changes had no significant effect on selected element concentrations on cow milk **(Erdogan, Celik and Erdogan, 2004)**. **Qu et al. (2018)** found relative high toxic metal levels from provinces with heavy industry. The average of milk exposure concentration for As, Pb, Cr, Hg, Al, and Ni was 1.35, 8.50, 34.58, 2.31, 284.16, and 10.78 μg.L^{-1}, respectively. Ni in milk is accumulated in fat.

This probably essential element was found in cow milk from Turkey in a concentration of 8.71 μg.kg^{-1} **(Totan and Filazi, 2020)** and from Spain 4 – 25 μg.kg^{-1} **(Llorent-Martínez et al., 2012)**.

The occurrence of contaminants in animal milk is connected with environmental quality. Metals are transferred from soil to water and/or feed to milk. **Zhou et al. (2019b)** note that different kinds of heavy metal contamination in raw milk may travel through complex pathways from the environment, directly or indirectly, via drinking water and soil. Heavy metals in silage may be the main contributor to milk contamination, as Pb, As, Cr, and Cd in silage all showed positive correlations with those in milk. The authors found that water may be the source of Pb and As in the milk, while Cr and Cd are transferred from the soil. The levels of elements in soil samples are shown in Table 3. The highest level of Ca, Cu, and Ni in soil was recorded in the strongly disturbed environment of the Čečejovce area. The source of metals may be an industrial activity (steel production, mining, waste combustion). The concentrations of Pb, Hg, Cd, As, Cu in the soil in this area exceeded the maximum limits in 2016 **(Juhasová et al., 2017)**. However, the concentrations of those metals

Table 2 Concentrations of essential and toxic elements in cow's milk in spring, summer and autumn in different environment.

Element	Undisturbed area (mg.kg^{-1} ±SD)	Moderately disturbed area (mg.kg^{-1} ±SD)	Strongly disturbed area (mg.kg^{-1} ±SD)
	Spring season (April)		
Ca	1294 ±11.402	981 ±92.898^{a**}	1098 ±98.590^{b*}
Cu	<0.05[1]	<0.05[1]	<0.05[1]
Fe	0.482 ±0.013	0.468 ±0.019	0.404 ±0.034$^{b** c**}$
Mg	114 ±2.646	85.4 ±5.550^{a***}	95.6 ±3.578$^{b*** c**}$
Se	<0.03[1]	<0.03[1]	<0.03[1]
Zn	4.24 ±0.195	3.44 ±0.182^{a***}	3.44 ±0.270^{b***}
As	<0.03[1]	<0.03[1]	<0.03[1]
Cd	<0.004[1]	<0.004[1]	<0.004[1]
Hg	<0.002[1]	<0.002[1]	<0.002[1]
Ni	<0.1[1]	<0.1[1]	<0.1[1]
Pb	<0.01[1]	<0.01[1]	<0.01[1]
	Summer season (July)		
Ca	1930 ±35.355	1426 ±160.873^{a**}	1570 ±93.005^{b***}
Cu	<0.05[1]	<0.05[1]	<0.05[1]
Fe	0.626 ±0.018	0.66 ±0.045	0.606 ±0.030
Mg	164.6 ±2.302	154 ±1.225^{a***}	160.2 ±7.014
Se	<0.03[1]	<0.03[1]	<0.03[1]
Zn	5.86 ±0.114	4.8 ±0.245^{a***}	5.48 ±0.460^{c*}
As	<0.03[1]	<0.03[1]	<0.03[1]
Cd	<0.004[1]	<0.004[1]	<0.004[1]
Hg	<0.002[1]	<0.002[1]	<0.002[1]
Ni	<0.1[1]	<0.1[1]	<0.1[1]
Pb	<0.01[1]	<0.01[1]	<0.01[1]
	Autumn season (September)		
Ca	1178 ±42.071	1214 ±25.010	1130 ±50.990^{c*}
Cu	<0.05[1]	< 0.05[1]	<0.05[1]
Fe	0.482 ±0.019	0.482 ±0.066	0.428 ±0.041^{b*}
Mg	92.8 ±3.114	103.8 ±2.950^{a***}	97 ±3.162^{c**}
Se	<0.03[1]	<0.03[1]	<0.03[1]
Zn	3.36 ±0.089	4.42 ±0.383^{a**}	3.64 ±0.195$^{b* c**}$
As	<0.03[1]	<0.03[1]	<0.03[1]
Cd	<0.004[1]	<0.004[1]	<0.004[1]
Hg	<0.002[1]	<0.002[1]	<0.002[1]
Ni	<0.1[1]	<0.1[1]	<0.1[1]
Pb	<0.01[1]	<0.01[1]	<0.01[1]

Note: *SD* – standard deviation; [a]differences between undisturbed area and moderately disturbed area; [b]differences between undisturbed area and strongly disturbed area; [c]differences between moderately disturbed area and strongly disturbed area; **p* <0.05; ***p* <0.01; ****p* <0.001; [1]Values below LOQ (limit of quantification).

recorded in our analyses were much lower or not detected (Cd).

Cd was also not detected in any of the milk samples collected dairies in the proximity of mines in Gauteng and North West Provinces of South Africa. This indicates the absence of Cd related toxicological risks in studied dairy farms **(Ataro et al., 2008)**. Arsenic concentration in soil samples was significantly highest (p <0.001) in the Tulčík area, Hg and Pb were found highest (p <0.001) in the Novoť area, Ni was significantly highest (p <0.001) in the Čečejovce area. The detailed biological cycle which causes the uptake of trace elements from the feed into milk or other dairy products is not well understood **(Herwig et al., 2011)**. **Rey-Crespo et al. (2013)** assume that the significantly higher As (65%) and Fe (13%) concentrations in cow milk found in the winter is probably related to higher consumption of concentration feed and soil ingestion when grazing. **Zhou et al. (2017)** also note that toxic and potentially toxic elements in raw milk were associated with those in feed and drinking water. Trace elements Mn, Fe, Ni, Ga, Se, Sr, Cs, U in water and Co, Ni, Cu, Se, U in feed were significantly correlated with those in milk. Moreover, the toxic and potentially toxic elements Cr, As, Cd, Tl, Pb in water and Al, Cr, As, Hg, Tl in feed were significantly correlated with those in milk. An analysis of cow's feed and milk at wastewater-irrigated agricultural farms in Pakistan revealed that contaminated fodder like maize and Brassica plants grown with wastewater and contaminated soil are common sources contributing the heavy metal contamination in raw milk **(Iqbal et al., 2020)**. Pb, Fe, Cu, and Zn were higher in the milk samples collected from the industrial region around Bursa, a province of Turkey, but no Hg was detected **(Simsek et al., 2000)**. Surprisingly, the TMR feed from strongly disturbed area contained significantly lowest concentrations (p <0.001) of all detected toxic and potentially toxic elements (As, Ni, Pb) in our analyses in spring. In autumn, the concentrations of these metals in feed were slightly higher in the Čečejovce area than in spring, but not the highest among the investigated areas. On the contrary, the „cleanest" area, officially classified as an area with an undisturbed environment, contained significantly highest (p <0.001) levels of As in spring and Pb (p <0.001) in spring and autumn in the feed. The occurrence of toxic metals in soil and feed did not affect their levels in cow milk as their concentrations in milk from all observed areas were under the LOQ. The concentrations of As in soil decreased in the order: moderately disturbed > undisturbed > strongly disturbed area. The same order was recorded in the feed in spring. The concentrations of Pb in soil and feed decreased in the same order: undisturbed > moderately disturbed > strongly disturbed area. The Ni concentration in soil in spring decreased in the order: strongly disturbed > undisturbed > moderately disturbed area, but in the feed, it decreased as follows: undisturbed = moderately disturbed > strongly disturbed area. The Hg concentration in soil in spring decreased in the order: undisturbed > strongly disturbed > moderately disturbed area but Hg in feed was under LOQ both in spring and autumn. These results confirm the variability of feed and soil contamination in different areas. Variable concentrations of Al, As, Ni, Hg, Pb, and Cd among most of the investigated regions of Egypt reported **Diab et al. (2020)**. The reason for these differences is probably different soil accumulation characteristics for different metals. Different soil accumulation characteristics for Cd, Cu, Zn, Pb, Ni, and Cr found **Zhang et al. (2018)**. Anyway, **Vidovic et al. (2005)** found a direct influence of atmospheric deposits on Cd and Zn distribution in the chain soil-cattle feed-milk. A significant decrease of the Cd and Zn concentrations in atmospheric deposits, originated mostly from vehicle traffic, resulting in a decrease of these metals in cow's feeds, milk, and soil in the Kikinda region, Serbia.

The concentrations of elements in feed analysed in three different regions are summarized in Table 4. Significant increases in milk Se concentration were observed with an increasing level of Se in the diet of cows and thus the concentration of Se in bovine milk is related to Se concentration in the feed **(Givens et al., 2004; Haug, Høstmark and Harstad, 2007)**. The Se content in the soil in Slovakia and Central Europe is generally low **(Ducsay, Ložek and Varga, 2009; Sager, 2006)** but there is a difference between the regions and soil types.

Table 3 Concentrations of essential and toxic elements in soil in spring in different environment.

Element	Undisturbed area (mg.kg^{-1} ±SD)	Moderately disturbed area (mg.kg^{-1} ±SD)	Strongly disturbed area (mg.kg^{-1} ±SD)
	Spring season (April)		
Ca	3930 ±45.826	3688 ±101.833^{a**}	10652 ±1015.071$^{b*** c***}$
Cu	25.92 ±0.853	16.54 ±1.029^{a***}	30.02 ±1.619$^{b** c***}$
Fe	26010 ±441.814	22908 ±203.887^{a***}	17840 ±392.747$^{b*** c***}$
Mg	4692 ±81.670	5102 ±68.337^{a***}	4490 ±70.0$^{b** c**}$
Se	0.308 ±0.008	0.242 ±0.037^{a*}	0.18 ±0.012$^{b*** c*}$
Zn	106.4 ±4.278	63.82 ±5.265^{a***}	59.9 ±1.061^{b***}
As	5.54 ±0.251	8.84 ±0.477^{a***}	3.32 ±0.148$^{b*** c***}$
Cd	<0.4[1]	<0.4[1]	<0.4[1]
Hg	0.069 ±0.002	0.049 ±0.002^{a***}	0.065 ±0.003$^{b* c***}$
Ni	21.54 ±0.329	20.88 ±0.335^{a*}	34.96 ±0.902$^{b*** c***}$
Pb	27.64 ±0.503	17.58 ±0.526^{a***}	15.7 ±0.406$^{b*** c***}$

Note: *SD* – standard deviation; [a] differences between undisturbed area and moderately disturbed area; [b]differences between undisturbed area and strongly disturbed area; [c]differences between moderately disturbed area and strongly disturbed area; *p <0.05; **p <0.01; ***p <0.001; [1]Values below LOQ (limit of quantification).

In Slovakia, the Se content in soil ranges from 0.04 to 0.80 mg.kg^{-1} but in the Nitra region, Se soil concentration exceeded the limit value 1.25-times **(Hegedusova et al., 2016)**. Our results (0.18 – 0.308 mg.kg^{-1}) show that Se in soil from all observed regions was in the range of average levels of Se in Slovakia. Very low (under the LOQ) concentrations of Se in milk is also caused by the low transfer of Se from soil to feed. In TMR feed, the Se concentrations were about 10-times lower than that in the soil in spring and slightly higher (up to 0.336 mg.kg^{-1}) in autumn. A similar trend was recorded in Cu concentration. Despite the fact, that Cu was under the LOQ in milk, we have found Cu in soil and feed samples from all investigated areas. The significantly highest Cu levels in soil were found in the strongly disturbed area in comparison to moderately disturbed (p <0.001) and undisturbed area (p <0.01). In feed, the significantly highest Cu concentration (p <0.001) was found in a moderately disturbed environment in spring and autumn in comparison to the undisturbed area. The limit value for soil varies by the soil type from 30 to 70 mg.kg^{-1} **(Regulation no. 508/2004)**. The concentration of Cu in the soil in Slovakia is 17 mg.kg^{-1} and at the cinnabar mine site, it ranges from 20.7 to 24.9 mg.kg^{-1} **(Kulikova et al., 2019)**. Our results are comparable to these values found in the country.

Soil Fe levels are not limited in Slovakia. Relatively high Fe concentrations were found in soil samples from all observed areas with significantly highest (p <0.001) concentration in the undisturbed area. **Khan et al. (2011)** reported a non-significant effect of sampling periods on soil Fe content, however, a higher transfer of Fe to pastures was found during October. Iron feed levels were higher in autumn in comparison to spring samples. The significantly highest (p <0.001) concentrations were found in the undisturbed area in spring and a moderately disturbed environment in autumn. Soil-derived elements like Fe may be ingested during grazing **(Orjales et al., 2018)** but we did not find an increase in Fe milk concentration above the normal levels.

Concentrations of Mg in soil did not differ between the spring and fall and ranged depending on the depth from 390 to 426 mg.kg^{-1} **(Hristov, Hazen and Ellsworth, 2007)**. About 10-times higher concentrations of Mg in soil samples were found in our study. The significantly highest (p <0.001) Mg level in soil was recorded in the moderately disturbed area in comparison to the undisturbed area of Novoť. Much higher concentrations of Mg (33.8 – 38.4 g.kg^{-1}) were found in contaminated soil at a magnesite mining region in China **(Wang et al., 2015)**. Concentrations of Mg in the soil are not limited in Slovakia. The feed contained generally higher Mg levels in autumn with the significantly highest levels in the moderately disturbed area which is in accordance with Mg levels in the soil. Plant Mg content in plant feeds varies between 0.7 and 3 g.kg^{-1} **(Haaranen, 2003)**. Our analyses show that the cow's feed contained around 3 g.kg^{-1}.

Table 4 Concentrations of essential and toxic elements in feed in spring and autumn in different environment.

Element	Undisturbed area (mg.kg^{-1} ±SD)	Moderately disturbed area (mg.kg^{-1} ±SD)	Strongly disturbed area (mg.kg^{-1} ±SD)
Spring season (April)			
Ca	2574 ±23.022	8412 ±397.706^{a***}	4844 ±367.872$^{b*** c***}$
Cu	4.36 ±0.207	12.3 ±0.667^{a***}	11.08 ±0.763$^{b*** c*}$
Fe	263 ±3.464	215 ±10.977^{a***}	223.8 ±18.226^{b**}
Mg	1558 ±14.832	3692 ±97.570^{a***}	1232 ±92.033$^{b** c***}$
Se	0.062 ±0.009	0.086 ±0.005^{a***}	0.021 ±0.003$^{b*** c***}$
Zn	30.82 ±1.711	83.26 ±1.358^{a***}	51.46 ±1.539$^{b*** c***}$
As	0.088 ±0.001	0.13 ±0.024^{a*}	0.025 ±0.003$^{b*** c***}$
Cd	<0.1[1]	<0.1[1]	<0.1[1]
Hg	<0.01[1]	<0.01[1]	<0.01[1]
Ni	1.48 ±0.084	1.48 ±0.130	0.192 ±0.042$^{b*** c***}$
Pb	0.56 ±0.012	0.506 ±0.042^{a*}	0.236 ±0.048$^{b*** c***}$
Autumn season (September)			
Ca	9938 ±470.394	7964 ±198.570^{a***}	10354 ±116.103^{c***}
Cu	12.82 ±1.132	25.98 ±1.043^{a***}	20.78 ±0.512$^{b*** c***}$
Fe	537.2 ±14.653	832 ±44.385^{a***}	441.8 ±31.444$^{b*** c***}$
Mg	3082 ±75.961	3308 ±21.679^{a**}	2952 ±101.341^{c**}
Se	0.053 ±0.003	0.25 ±0.029^{a***}	0.336 ±0.019$^{b*** c***}$
Zn	57.04 ±1.278	128 ±1.871^{a***}	127.8 ±3.114^{b***}
As	0.153 ±0.040	0.184 ±0.023	0.166 ±0.015
Cd	<0.1[1]	<0.1[1]	<0.1[1]
Hg	<0.01[1]	<0.01[1]	<0.01[1]
Ni	1.42 ±0.084	3.14 ±0.321^{a***}	1.82 ±0.192$^{b** c***}$
Pb	0.48 ±0.012	0.308 ±0.019^{a***}	0.39 ±0.016$^{b*** c***}$

Note: *SD* – standard deviation; [a]differences between undisturbed area and moderately disturbed area; [b]differences between undisturbed area and strongly disturbed area; [c]differences between moderately disturbed area and strongly disturbed area; *p <0.05; **p <0.01; ***p <0.001; [1]Values below LOQ (limit of quantification).

These concentrations agree with the soil Mg levels and did not cause the elevation of Mg in raw cow milk.

Zn concentration in bovine milk is significantly affected by the dietary intake of fat and the transfer of fat from diet to milk might facilitate the transfer of Zn from diet to milk. **(Wiking, Larsen and Sehested, 2008)**. The authors also found a similar trend in the variability of Zn levels in the feed as we have found in our analyses. The significantly highest (p <0.001) Zn concentration in feed was found in spring and autumn in a moderately disturbed area and in a strongly disturbed area. These findings may correspond to the mining and industrial (metallurgy) activities in these areas and using of pesticides containing metals like Zn. Mean Zn concentrations in soil in the cinnabar mining site in Slovakia found by **Kulikova et al. (2019)** ranged from 58.1 to 61.6 $mg.kg^{-1}$, which are comparable to levels in disturbed areas in our study. However, the limit value for soil Zn by the soil type ranges from 100 to 150 $mg.kg^{-1}$ **(Regulation no. 508/2004)** and our results show Zn levels in the soil below the lowest permitted limit in disturbed areas. On the contrary, the significantly highest (p <0.001) Zn concentration in soil was found in the undisturbed area. The concentrations of Zn in soil and feed do not correspond. Lower levels of Zn in soil recorded **Khan et al. (2006)** in Pakistan, but **Baranowska, Barchańska and Pyrsz (2005)** found Zn content in the soil in the higher range of 9.15 – 424.5 $\mu g.g^{-1}$ in Poland.

CONCLUSION

The positive findings of this study are, that the concentrations of all toxic elements in milk were under the limits of quantification from all investigated areas regardless of the environmental contamination level. We found seasonal variations in occurrence of essential elements with the highest levels in summer. The significantly highest levels of some elements in soil were recorded in the undisturbed environment. In feed, the higher concentrations almost all elements were found in the autumn. Higher levels of some elements in soil did not cause their elevation in the feed and milk. The milk from the investigated areas was not contaminated with the elements posing a health risk for consumers and it is considered safe for human consumption. Despite the fact, that all concentrations of analyzed elements were under the permissible limits, there is a constant need to monitor an environmental burden of metals in the different, even undisturbed regions of Slovakia with animal production to recognize another, hidden sources of metal contamination that may impact the food chain and human health.

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PROCEDURES FOR THE IDENTIFICATION AND DETECTION OF ADULTERATION OF FISH AND MEAT PRODUCTS

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ABSTRACT

The addition or exchange of cheaper fish species instead of more expensive fish species is a known form of fraud in the food industry. This can take place accidentally due to the lack of expertise or act as a fraud. The interest in detecting animal species in meat products is based on religious demands (halal and kosher) as well as on product adulterations. Authentication of fish and meat products is critical in the food industry. Meat and fish adulteration, mainly for economic pursuit, is widespread and leads to serious public health risks, religious violations, and moral loss. Economically motivated adulteration of food is estimated to create damage of around € 8 to 12 billion per year. Rapid, effective, accurate, and reliable detection technologies are keys to effectively supervising meat and fish adulteration. Various analytical methods often based on protein or DNA measurements are utilized to identify fish and meat species. Although many strategies have been adopted to assure the authenticity of fish and meat and meat a fish products, such as the protected designation of origin, protected geographical indication, certificate of specific characteristics, and so on, the coverage is too small, and it is unrealistic to certify all meat products for protection from adulteration. Therefore, effective supervision is very important for ensuring the suitable development of the meat industry, and rapid, effective, accurate, and reliable detection technologies are fundamental technical support for this goal. Recently, several methods, including DNA analysis, protein analysis, and fat-based analysis, have been effectively employed for the identification of meat and fish species.

Keywords: food fraud; adulteration; detection method; protein technologies; DNA technologies

INTRODUCTION

At present, there is no harmonized definition of food fraud in the European Union (EU) 2017. However, it is commonly accepted that the term 'food fraud' covers any violation of food law that is an intentional and deceptive misrepresentation of food for financial gain **(van Ruth et al., 2017; EC, 2019.** Food fraud is about "any suspected intentional action by businesses or individuals to deceive purchasers and gain undue advantage therefrom. **Spink and Moyer (2011)** have elaborated on this definition and describe seven types of food fraud: adulteration, tampering, over-run, theft, diversion, simulation, and counterfeit. These intentional infringements to the EU agri-food chain legislation may hinder the proper functioning of the internal market and may also constitute a risk to humans. However, existing databases that monitor food fraud Such as the Rapid Alert System for Food and Feed (RASFF) and HorizonScan have their categorizations **(Bouzembrak et al., 2018)**. RASFF has six categorizes for fraud (Improper, fraudulent, missing or absent health certificates; illegal importation; tampering; improper, expired, fraudulent or missing common entry documents or import declarations; expiration date; mislabeling) as does HorizonScan (adulteration/substitution, fraudulent health certificate/documentation, produced without an inspection, unapproved premises, expiry date changes). Four key operative criteria are referred to for distinguishing whether a case should be considered as fraud or as non-compliance: if a case matches all four criteria, then it could be considered a suspicion of fraud: violation of EU rules, deception of customers, undue advantage and intention. Meat and fish are food categories that are highly vulnerable to adulteration. Although there are various national and international laws for supervising the quality and safety of fish, meat, and meat and fish products, meat adulteration is still widespread. Most meat adulteration is economically motivated, such as the low-cost addition of duck meat and fish to mutton **(Wang et al., 2019a),** which causes consumers to suffer economic losses. Meat and fish adulteration may lead to serious public health risks, such as exposure to toxins, pathogens, or allergens in these products **(Magiati et al., 2019; Spink and Moyer, 2011)**.

MEAT AND FISH ADULTERATIONS

The demand for meat and fish products is high and as a result, meat is one of the most highly-priced food commodities; therefore, a prime target for food fraud **(Cawthorn et al., 2013)**. The examples of adulteration are presented in Table 1.

Table 1 Scholarly reports on fish and meat ingredient fraud and analytical methods for detection.

Ingredient Category	Ingredient	Adulterant	Type of fraud	Publictation year	Reported detection method and reference
Meats	Chicken meat (cornfed)	Chicken meat fromnon cornfed chickens	Replacement	2010	IRMS (13C/12C) on extracted protein and lipid fractions of meat **(Rhodes et al., 2010)**
Meats	Meat products	Chickpea flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha et al., 2009)**
Meats	Meat products	Pea flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha a et al., 2009)**
Meats	Meat products	Rice flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha et al., 2009)**
Meats	Meat products	Soy flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha et al., 2009)**
Meats	Minced meat (beef)	Ox offal tissue (kidney or liver)	Replacement	1999	MIR with chemometrics **(Al-Jowder et al., 1999)**
Meats	Minced meat (chicken, pork, or turkey)	Meat from non-authentic species	Replacement	1999	MIR with chemometrics **(Al-Jowder et al., 1999)**
Meats	Processed meat product	Soybean protein	Replacement	2005	Perfusion reversed phase chromatography with UV detection on extracted protein for adulterant marker detection **(Castro- Rubio et al., 2005)**
Seafood	Anglerfish	Anglerfish of non-authentic species	Replacement	2008	Review of methods: HPLC-MS/MS, ELISA, dients ed compounds extractive electrospray ionization timeofflight MS, and GC-MS **(Tittlemier, 2010)**
Seafood	Canned tuna	Bonito (*Euthynnus affinis*)	Replacement	1996	Sequence and restriction site analysis of PCR mitochondrial DNA **(Ram, Ram and Baidoun, 1996)**
Seafood	Canned tuna	Frigate mackerel (*Auxis thazard*)	Replacement	1996	Sequence and restriction site analysis of PCR mitochondrial DNA **(Ram, Ram and Baidoun, 1996)**

Table 1 Scholarly reports on fish and meat ingredient fraud and analytical methods for detection. Continue.

Ingredient Category	Ingredient	Adulterant	Type of fraud	Publictation year	Reported detection method and reference
Seafood	Crab (species specific)	Crustacean of non- authentic species	Replacement	2007	UV-Vis spectrometry with chemometrics **(Gayo and Hale, 2007)**
Seafood	Crab meat	Surimi-based artificial crab meat	Replacement	2006	UV-Vis spectrometry with chemometrics **(Gayo and Hale, 2006)**
Seafood	Eel	Fish of non-authentic species	Replacement	2008	DNA based method using fluorogenic ribonuclease protection assay to detect single nucleotide polymorphisms **(Kitaoka et al., 2008)**
Seafood	Fish	Melamine	Replacement	1982	Wet-chemical method with UV detection **(Cattaneo and Cantoni, 1982)**
Seafood	Fish	Non-authentic species	Replacement	2001	Isoelectric focusing electrophoresis for protein fingerprinting **(Etienne et al., 2001)**
Seafood	Grouper (*Epinephelus guaza*)	Wreck fish (*Polyprion americanus*) or Nile rRNA gene by PCR followed by single perch (Lates strand conformational polymorphism niloticus)	Replacement	2001	DNA analysis using mitochondrial 12S rRNA gene by PCR followed by single strand conformational polymorphism analysis **(Asensio et al., 2001a)**
Seafood	Prawns	Crustacean of non-authentic species	Replacement	2008	PCR **(Pascoal et al., 2008)**
Seafood	Scampi (*Neplirops norvegicus*)	Crustacean of non-authentic species	Replacement	1995	SDS electrophoresis on protein extract **(Craig, Ritchie and Mackie, 1995)**

Uncovering of adulterated meat products is important for several reasons. Allergic individuals and those who hold religious beliefs that specify allowable intake of certain species have a special interest in proper labeling. Proper labeling is also important to help fair-trade. The need for analytical species-specific methods is clearly illustrated by the following examples: **Hsieh, Chai and Hwang (2007)** found, with the use of immunoassays, meat from undeclared animal species in 15.9% of cases in raw products and 22.9% of cases in cooked products analyzing a total of 902 meat products. In a more recent investigation performed on 100 meat products, also with the use of immunoassays, meat from undeclared species was found in 22.0% of cases, primarily with poultry substituting beef **(Ayaz et al., 2006)**. The provenance of food, especially meat products, is a sensitive topic but there are tools available to support producers in demonstrating compliance with legislators and other authorities. Since the level of awareness about food quality and safety has recently increased, food fraud has become a major global issue. Hence, the identification of meat and fish products adulteration with unfavorable and inappropriate animal species is important from health, economic, and religious points of view **(Mousavi et al., 2015)**. Currently, the protein-based techniques (e.g. electrophoresis, isoelectric focusing, ELISA, and chromatography) have been utilized for meat and fish adulteration. These methods are laborious, expensive, and sophisticated instrumentation

with great technical proficiency **(Calvo et al., 2002, von Bargen et al., 2014)**.

Numerous analytical techniques which rely on protein analysis have been developed for fish species identification: electrophoretic techniques such as isoelectric focusing or SDS-PAGE **(Ataman, Celik and Rehbein, 2006, Mackie et al., 2000)**; chromatographic techniques **(Horstkotte and Rehbein, 2003, Knuutinen and Harjula, 1998)** and immunological techniques such as immunodiffusion and ELISA **(Fernández et al., 2002a, Ochiai et al., 2001)**. Therefore, the development of advanced detection methods constitutes an important first line of defense for both detecting and deterring food fraud **(Moore, Spink and Lipp, 2012)**. Although most of these methods are of considerable value in certain instances, they are not suitable for routine sample analysis because proteins lose their biological activity after animal death, and their presence and characteristics depend on the cell types. Furthermore, most of them are heat-labile. Thus, for fish species identification in heat-processed matrices, a DNA method rather than protein analysis is preferable **(Lockley and Bardsley, 2000)**.

DNA TECHNOLOGIES

As a prerequisite for accurate species quantification, DNA has to comply with minimum requirements about yield, purity, and integrity. Yield is an important parameter since food DNA has to be in a sufficient amount to allow the reliable and repeatable downstream analysis of meat species **(Heydt et al., 2014)**. The concentration and purity of DNA extracts are critical factors dominating the results of real-time PCR. DNA quantification is typically measured by either spectrophotometric or fluorometric methods, with the former representing the most commonly used technique **(Costa et al., 2017)**. DNA integrity determines the fraction of DNA that can be amplified by PCR **(Gilbert et al., 2007)** and it can be evaluated based on the average size distribution of fragmented DNA. Although often underestimated, DNA isolation is a crucial step for molecular analysis of food due to its heterogeneity in terms of composition and processing. The presence of chemical inhibitors, proteins, and/or damaged DNA are common situations in meat food analyses. Moreover, the extraction methods themselves can further influence the yield, purity, and integrity of DNA depending on the type of food matrix **(Şakalar et al., 2012)**. The final consequence is that the amount of species DNA determined in the product would not reflect the real amount in the source material, impairing quantitative measurements **(Primrose et al., 2010)**. DNA exists in all tissues of individual animals and is more conserved than proteins **(Kumar et al., 2015; Xiang et al., 2017)**. More importantly, DNA fragments have shown better thermal stability than that of proteins in processed meat, so they could be chosen as markers for authenticity determination in processed meat **(Kaltenbrunner, Hochegger and Cichna-Markl, 2018; Kang and Tanaka, 2018; Kumar et al., 2015; Ruiz-Valdepeñas Montiel et al., 2017; Xu et al., 2018)**. Of the different DNA markers used for fish species identification, mitochondrial DNA (mtDNA) possesses several advantages over nuclear DNA for studies of speciation in fish products. It is relatively more abundant in total nucleic acid preparations than nuclear DNA, with the copy number of the mitochondrial genome exceeding that of the nuclear genome several folds **(Alberts et al., 1994)**. Research on fish mitochondrial DNA (mtDNA, mitogenome) has led to substantial advances in the fields of species authentication and population biology **(Miya et al., 2001)**. Mitochondrial DNA tends to be maternally inherited so that individuals normally possess only one allele and thus sequence ambiguities from heterozygous genotypes are generally avoided. The relatively high mutation rate compared to nuclear genes has tended to result in the accumulation of enough point mutations to allow the discrimination of even closely related species. It should however be noted that mitochondrial DNA also exhibits a degree of intraspecific variability and so care has to be taken when studying differences between organisms based on single base polymorphisms **(Chow and Inogue, 1993)**. However, the use of nuclear markers may be useful for fish species discrimination because of the existence of introns of different sizes which allow sometimes the amplification of species-specific DNA fragments **(Ferguson et al., 1995)**. The comparative analysis of the commonly applied meat adulteration DNA techniques is present in Table 2.

Polymerase chain reaction-restriction fragment length polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a technique for variation analysis by using restriction endonuclease digestion to identify specific sequences of conserved regions of DNA amplified by using PCR. PCR-RFLP is a sensitive, accurate, and versatile method for meat authenticity verification **(Hsieh, Chai and Hwang, 2007; Rashid et al., 2015)**, and more simple and time-saving than real-time PCR **(Ali et al., 2018)**. The result is that each meat species displays its typical restriction profile **(Fajardo et al., 2006)**. Several studies have demonstrated that LAMP might be a fast, efficient, and economical method for meat adulteration detection **(Azam et al., 2018; Cho et al., 2014; Deb et al., 2016; Ran et al., 2016; Sul, Kim and Kim, 2019; Wang et al., 2019b; Xu et al., 2017; Zhang et al., 2019)**. Using LAMP combined with colorimetric detection technology for the COI gene, 0.1% of horse meat could be detected from processed meats **(Wang et al., 2019a)**.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a newly developed meat adulteration identification technology based on DNA markers in recent years **(Lee et al., 2016; Zhang, Lowe and Gooding et al., 2014)**. LAMP is simple and easy to perform once the appropriate primers are prepared, requiring only four primers, a DNA polymerase, and a regular laboratory water bath or heat block for reaction **(Notomi et al., 2000)**.

PCR

The direct PCR method has the characteristics of high sensitivity, high resolution, and specificity, so it is commonly used in meat authenticity and origin traceability **(Bhat et al., 2016; Ha et al., 2017)**. **Ha et al. (2017)** developed species-specific PCR methods of the mitochondrial D-loop to detect pork adulteration in

commercial beef and/or chicken products, and the methods were able to detect as little as 1% pork in heat-treated pork-beef-chicken mixtures. However, the conventional single-species PCR method could only detect one specific species of adulterant in products **(Kumar et al., 2015)**, which is of low commercial value because there might be many other adulterants in the products. This method provides very accurate and reproducible quantitation of gene copies. Unlike other quantitative PCR methods, real-time PCR does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and resulting in much faster and higher throughput assays **(Heid et al., 1996)**. Multiplex PCR assays with multiple species-specific primers have been greatly developed since they offer multiple target detection in a single reaction **(Ali et al., 2015; Böhme et al., 2019; Dai et al., 2015; Hou et al., 2015)**. PCR-SSCP has proved successful for the identification of fishery products such as salmon, trout, eel, and sturgeon **(Rehbein et al., 1997)**, canned tuna species **(Rehbein et al., 1999, Weder et al., 2004)**, flatfish species **(Céspedes et al., 1999)**, grouper, Nile perch and wreckfish fillets **(Asensio et al., 2001b)**, clam species **(Fernández et al., 2002b)** and codfish **(Comi et al, 2005)**, among others.

PCR-RFLP

In PCR-RFLP, a conserved region of the DNA sequence is amplified using PCR, followed by digestion with restriction enzymes, which can reveal genetic variation between species **(Partis et al., 2000)**. In a search for fast and simple genetic techniques, PCR-RFLP has gained acceptance among fish species identification methods, since it is much easier to perform and less costly than conventional DNA sequencing and nucleotide sequence analysis **(Meyer et al., 1995)**. This method has been used for the discrimination of mackerel species **(Arahishi, 2005)**, commercial canned tuna species **(Lin and Hwang, 2007, Pardo and Pérez-Villarea, 2004)**, eel species **(Rehbein et al., 2002)**, flatfish species **(Céspedes et al., 1998, Comesaña et al., 2003)**, cephalopod mollusks **(Colombo et al., 2002)**, or different processed fish products **(Akasaki et al., 2006, Chakraborty et al., 2007, Hsieh, Chai and Hwang, 2007)**.

Real-time PCR

Real-time PCR is performed by monitoring the fluorescence signal, which allows for deducing the initial quantity of the target genes without additional steps **(Xu et al., 2018)**. The real-time PCR method has a very large dynamic range of starting target molecule determination (at least five orders of magnitude). Real-time quantitative PCR is extremely accurate and less labor-intensive than current quantitative PCR methods **(Heid et al., 1996)**. SYBR Green and TaqMan technology are commonly used in quantitative methods (the working principle is outlined in the review of **Kumar et al., 2015**). SYBR Green technology can only detect a single species, but the detection cost was lower than that of TaqMan technology. **Li et al. (2019)** developed a novel reference primer-based mitochondrial 12S rRNA for the quantitative determination of goat meat adulterated with pork by using real-time PCR. The method showed high specificity and sensitivity for goat meat mixed with pork within the 10% to 100% mixture-level range. TaqMan technology has higher specificity and sensitivity than those of SYBR Green technology. More importantly, it can be used for multispecies detection **(Xu et al., 2018)**.

Droplet digital PCR

Droplet digital PCR (ddPCR) is a new method for nucleic acid detection and quantification. The principle of this method is to perform independent PCR on a large number of small reactors in the form of droplets that contain or do not contain one copy of the target molecule template in each reactor, to achieve "single-molecule template PCR amplification" **(Cai et al., 2017; Li et al., 2018a; Pohl and Shih Ie, 2004)**. After amplification, the number of copies of the target sequence can be counted by the number of positive reactors based on the fluorescence signal.

RAPD

The RAPD technique involves PCR amplification with a single primer to generate a collection of DNA fragments or fingerprint, which is expected to be consistent for the same primer, DNA, and conditions used **(Williams et al., 1990)**. This technique has been used for the discrimination of populations of Hilsa shad **(Dahle et al., 1997)**, species of Anguilla **(Takagi and Taniguchi, 1995)**, tilapia fish species and subspecies **(Bardakci and Skibinski, 1994)**, species of the genus Barbus **(Callejas and Ochando, 2001)**, grouper, Nile perch and wreckfish **(Asensio et al., 2002)**, salmonids **(Jin et al., 2006, Yamazaki et al., 2005)**, among others **(Dinesh et al., 1993, Partis and Wells, 1996)**. The main advantages of RAPD are (i) it does not require previous knowledge of DNA sequences of the species under study and (ii) it targets many sequences in the DNA of the sample, producing DNA patterns that allow comparison of many loci simultaneously. However, RAPD analysis presents some disadvantages: (i) it may not be practical to identify the species of origin in products containing mixtures of species **(Martínez and Malmheden Yman, 1998)** and (ii) it does not seem to be adequate for analysis of severely degraded material, as in autoclaved samples **(Martínez and Malmheden Yman, 1998)**.

DNA barcoding and next-generation sequencing

The above reviewed DNA-based technologies are mainly targeted detection methods, but in meat adulteration detections, many unknown meat species should be identified **(Cottenet et al., 2020)**. Following this need, an untargeted detection technology named DNA barcoding had been developed **(Cavin et al., 2018; Hebert et al., 2003)**. DNA barcoding is particularly successful when applied to seafood because of several reasons:

i) in comparison to other animal sources (e.g. cattle, sheep, goat, horse) the number of species is higher, so the effectiveness of the technique is enhanced;

Table 2 Comparative analysis of the commonly applied meat adulteration DNA techniques.

Techniques	Specificity	Sample preparation	Detection time	Multispecies detection	Operator requirements	Detection costs	Commercial availability	Application locations
Direct PCR	High but vulnerable	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Time-consuming	Yes	Professional	High	Commercial kits available	Lab
Real-time PCR	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Time-consuming	Yes	Professional	High	Commercial kits available	Lab
PCR-RFLP	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Time-consuming	Yes	Professional	High	Commercial kits available	Lab
LAMP	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Less time-consuming	Yes	Professional	High	Commercial kits available	Lab or onsite
Protein mass	High	Sample ground→ protein extraction→ purification→ digestion	Time-consuming	Yes	Professional	High	No	Lab
ddPCR	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Less time-consuming	Yes	Professional	High	No	Lab
A barcoding	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Less time-consuming	Yes	Professional	High	Public databases available (BOLD)	Lab
ELISA	High	Sample ground→protein extraction→ quantification	Less time-consuming	No	Simple training	Low	Commercial kits available	Lab or onsite
Protein immunosensor	High	Sample ground→protein extraction→ quantification	Less time-consuming	No	Simple training	Low	No	Lab or onsite

ii) classical identification approaches are not useful in many cases (following industrial processing, morphological characteristics are often lost and classical identification processes are no longer effective) and

iii) identification can often proceed beyond species level, allowing the identification of local varieties and hence the origin of the product. Through PCR amplification and sequencing of specific gene fragments, and then search it in the Barcode of Life Data (BOLD) system and the U.S. National Center for Biotechnology Information database, the adulterated meat species could be identified **(Fiorino et al., 2018)**. The early DNA barcoding technology mainly relied on Sanger DNA sequencing for an approximately 650 bp region of COI and the CtyB gene of the animal species **(Böhme et al., 2019)**. DNA Barcoding application can be applied to authenticate labeling and certification labels. This technique has aided several researchers in discovering mislabeled/substitution incidences, for example, **Filonzi, et al., (2010)** found halibut were substituted with pangasius However, when there are multiple adulterated ingredients in meat products, the traditional Sanger sequencing will generate multiple or overlaying sequencing peaks, resulting in false sequence information. Therefore, a DNA metabarcoding method had been constructed to implement multispecies identification in complex samples using next-generation sequencing (NGS) technology. Furthermore, for processed meat products, DNA can be degraded to small fragments (<200 bp) depending on the treatment **(Cavin et al., 2018)**. Thus, a mini-barcoding method, which focuses on shorter DNA fragments (100 to 200 bp), had been developed by using NGS technology **(Böhme et al., 2019; Hu et al., 2018)**. Compared to the early DNA barcoding technology, mini-barcoding has the advantages of higher throughput and higher sensitivity **(Böhme et al., 2019; Hu et al., 2019)**. Also, it is applicable for meat identification even on highly processed meat products when targeting small fragments **(Cottenet et al., 2020).** Recently, **Cottenet et al. (2020)** successfully applied a commercial NGS Food Authenticity Workflow to identify untargeted meat species, 46 pure and mixture meat species were successfully tested, including some close-related species, such as bison versus beef and red deer versus reindeer. Furthermore, the method was also suitable for processed (grounded, cooked, and canned) samples identification. However, DNA barcoding technology also has some disadvantages, such as expensive sequencing costs, time-, and sample-consuming **(Fiorino et al., 2018)**.

PROTEIN TECHNOLOGIES

Meat adulteration detection by using PCR methods is usually affected by many factors, such as poor trace quantitative analysis, sampling pollution, and DNA degradation in meat processing **(Di Pinto et al., 2015; Li et al., 2018a; Naveena et al., 2017)**. Moreover, DNA extraction is time-consuming and must be optimized for each particular case to ensure that enough DNA was obtained for the analysis **(Song et al., 2017)**. Protein is the main component of meat. The specific protein composition and three-dimensional structure of specific proteins have certain conservation and specificity between species, which is suitable for meat adulteration detection. Moreover, some protein molecules are tissue-specific and can be used for the identification of less valuable additives, such as connective tissue, blood plasma, or milk preparations **(Jiang et al., 2018; Montowska and Spychaj, 2018; Ofori and Hsieh, 2015)**. The comparative analysis of the commonly applied meat adulteration protein techniques is present in Table 3.

Enzyme-linked immunosorbent assay

EIA/ELISA uses the basic immunology concept of an antigen-binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins, peptides, hormones, or antibodies in a fluid sample. There are two kinds of immunoassay techniques used in meat adulteration detection: enzyme-linked immunosorbent assay (ELISA) and immunosensors. ELISA is the most widely applied immunoassay method of meat adulteration detection **(Thienes et al., 2018)**. The commonly used ELISA methods for meat adulteration detection are direct ELISA **(Mandli et al., 2018; Seddaoui and Amine, 2020)**, sandwich ELISA **(Ayaz et al., 2006; Hsieh and Ofori, 2014; Thienes et al., 2018; Zvereva et al., 2015)**, and indirect competitive ELISA **(Hsieh and Ofori, 2014; Jiang et al., 2018; Mandli et al., 2018)**. Compared to DNA-based detection technologies, ELISA methods show the simplicity of sample preparation, low cost, and less time consumption. Also, ELISA detection does not require complex equipment and is easily feasible for onsite monitoring **(Mandli et al., 2018; Thienes et al., 2019)**.

Immunosensors

However, immune techniques are characterized by their simplicity of sample preparation, absence of the need for complex equipment and qualified personnel, and high productivity of serial testing. As well, for food authentication, electrochemical immunosensors are an alternative detection tool and are highly feasible for on-site usage; therefore, there is only one previously reported immunosensor for meat authentication **(Lim and Ahmed, 2016)**. The principle of immunosensor methods is similar to that of ELISA methods, but the former uses a biosensor to transmit and amplify the optical, electrical, or other signals of the immune response to a detectable signal, so the sensitivity of the method is better than that of ELISA. The immunosensor technique has been widely used in food allergy, pesticide residue, and milk adulteration analyses, among others. However, only a few reports have utilized immunosensing for meat adulteration detection **(Kuswandi et al., 2017; Lim and Ahmed, 2016; Mandli et al., 2018; Masiri et al., 2016)**.

Protein mass spectrometry analysis

Modern mass spectrometers can accurately measure thousands of compounds in complex mixtures over a given liquid chromatography method, depending on the desired outcome and method duration. This stream of analytical chemistry has wide-ranging applications across food, pharma, environmental, forensics, clinical, and research **(Broadbent et al., 2020)**. Recently, mass spectrometry technologies based on protein and peptide analysis have rapidly evolved and have been increasingly applied for meat species identification.

Table 3 Comparative analysis of the commonly applied meat adulteration protein techniques.

Detection items	Detection technology	Immunogen and antibody	Method sensitivity(limit of detection)	References
Pork adulteration in beef	Direct ELISA	Porcine immunoglobulins G (IgG) and polyclonal antibodies	0.01% (w/w) of pork in beef	**Seddaoui and Amine (2020)**
Pork adulteration in meat	Indirect competitive ELISA	Porcine IgG and polyclonal antibodies	0.1% of pork adulteration	**Mandli et al. (2018)**
Porcine hemoglobin in meat products	Indirect competitive ELISA	Mammalian hemoglobin 13F7 and monoclonal antibodies (MAbs 13F7)	0.5 ppm of P_{Hb}	**Jiang et al., (2018)**
Pork fat protein in other animal meats	Indirect ELISA	Thermal stable-soluble protein (TSSP) and monoclonal antibodies (MAbs PF 2B8-31)	1% (w/w) of pork fat adulteration	**Kim et al. (2017)**
Fat adulteration in cooked and noncooked of pork, beef, and chicken	Indirect ELISA	Skeletal muscle troponin I (smTnI) and monoclonal antibodies (commercial ab97427)	ND	**Park et al. (2015)**
Cooked wild rat meat in pork, beef, and chicken	Sandwich ELISA	Rat heat-resistant proteins and polyclonal antibodies	0.01 μg/L based OD values	**Chen et al., (2020)**
Heated mammalian meats adulterated in poultry meats	Sandwich ELISA	Mammalian skeletal troponin and monoclonal antibodies (MAbs 6G1 and 8F10)	1% (g/g) of heated meats adulterated in poultry meats	**Jiang et al., (2020)**
Cooked beef in the pork, horse, chicken, goat, and sheep meat	Sandwich ELISA	ND	0.1% (w/w) of the cooked products	**Thienes et al., (2019)**
Cooked chicken/turkey in pork, horse, goat, or sheep meat	Sandwich ELISA	ND	0.1% (w/w) of the cooked products	**Thienes et al., (2019)**
Pork is cooked horse, beef, chicken, goat, and lamb meats	Sandwich ELISA	ND	0.1% (w/w) for cooked samples	**Thienes et al. (2018)**
Wheat protein in ground chilled pork and beef mixture	Sandwich ELISA	Gliadin and monoclonal antibodies	1% (w/w) for spiked samples	**Petrášová et al. (2017)**
Soybean proteins in surimi products	Sandwich ELISA	Soybean trypsin inhibitor (STI) and monoclonal antibodies	13.6 mg/kg samples	**Jiang et al. (2015)**
Mammalian muscle tissues in raw meat and meat products	Sandwich ELISA	Skeletal muscle protein troponin I (TnI) and monoclonal antibodies	4.8 ng/mL of bovine TnI	**Zvereva et al. (2015)**
Pork adulteration in beef meatballs	Electrochemical immunosensor	Porcine IgG and polyclonal antibodies	0.01% of pork adulteration	**Mandli et al. (2018)**
Pork adulteration in cooked meatballs	Lateral flow immunosensor	Porcine IgG and polyclonal antibodies	0.1% (w/w) for pork in beef meatballs	**Kuswandi et al. (2017)**
Horse meat adulteration in meat products	Lateral flow immunosensor	Horse serum albumin (HSA) and polyclonal antibodies	0.01% and 1.0% adulteration for raw and cooked horse meat	**Masiri et al. (2017)**
Pork adulteration in raw meat	Label-free electrochemical immunosensor	Porcine serum albumin (PSA) and polyclonal antibodies	0.5 pg/mL PSA in buffer solution	**Lim and Ahmed (2016)**

Table 3 Comparative analysis of the commonly applied meat adulteration protein techniques. Continue.

Detection items	Detection technology	Immunogen and antibody	Method sensitivity(limit of detection)	References
Bovine adipose tissue in meat products	Label-free electrochemical immunosensor	Ruminant-specific muscle protein and polyclonal antibodies	2% bovine fat-in-pork fat 1% bovine fat-in-porcine meat-and-bone meal 0.5% bovine fat-in-soy meal mixtures	**Hsieh and Gajewski (2015)**
Duck, goose, and chicken in processed meat products	LC–ESI–QTOF–MS LC–ESI–QQQ–MS/MS	Hemoglobin alpha for duck: FMCAVGAVLTAK Hemoglobin beta for goose: FFSSFGNLSSPTAILGNPMVR Myosin-binding protein C for chicken: LDVPISGEPAPTVTWK	ND	**Fornal and Montowska (2019)**
Grain proteins adulteration into meat products	HPLC-MS/MS	Barley: IETPGPPYLAK, Oat: DFPITWPWK, Rice: ELGAPDVGHPMSEVFR, Rye: TPFASTVAGIGGQ, Wheat: SVAVSQVAR	Oats and rye: 5 mg/kg meat product; barley and wheat: 10 mg/kg meat product	**Jira and Munch (2019)**
Porcine blood plasma to emulsion-type pork sausages	UHPLC-MS/MS	Plasma peptide marker of ISEPLATETVR GSLDEFFHR, ISPLPDITPADFK, DPFPDFFSPVLK	0.7% (w/w) meat substitution by porcine plasma	**Stader et al., (2019)**
Shrimp species in seafood	SWATH-MS	Myosin heavy chain type a for *Marsupenaeus japonicas*: AAVELDDLHASAER Arginine kinase for *Fenneropenaeus Chinensis*: GTYYPLTGMGK Sarco/endoplasmic reticulum Ca^{2+}-ATPase for *Litopenaeus vannamei*: IGVFGENEETAGK	ND	**Hu et al. (2018)**
Pork, beef, lamb, chicken, duck, soy, peanut, and pea adulteration in meat products	UPLC-MS/MS	Conglutin/Ara h 6 for peanut: EIMNIPQQCNFR, Alpha subunit of beta conglycinin for soy: ESYFVDAQPK, P54 protein for pea: GIIGLVAEDR, Myoglobin for duck: HGVTVLTQLGK, Creatine kinase M-type for chicken: DLFDPVIQDR, Hemoglobin subunit beta for sheep: VDEVGAEALGR, Carbonic anhydrase 3 for beef: LVNELTEFAK, Hemoglobin subunit beta for pig: VNVDEVGGEALGR	0.5% adulterations of any of the eight species	**Li et al. (2018b)**
Horse, pork, and beef meat in smoked sausages	Infusion MS	Myosin-1 for pork: SALAHAVQSSR, Myoglobin for beef: HPSDFGADAQAAMSK, Myoglobin for horse: VEADIAGHGQEVLIR	5% (w/w) for pork and beef in the three-component matrix and 1% (w/w) for horse meat	**Montowska and Spychaj (2018)**

Table 3 Comparative analysis of the commonly applied meat adulteration protein techniques. Continue.

Detection items	Detection technology	Immunogen and antibody	Method sensitivity (limit of detection)	References
Duck, pig, cattle, chicken, and sheep in cooked meats	UPLC-TripleTOF-MS UPLC-MS/MS	M-protein, striated muscle for chicken: FWIQAESLSPNSTYR, Alpha-enolase for duck: LMLDMDGSENK, Trifunctional enzyme subunit alpha (mitochondrial) for pig: FAGGNLDVLK, Stress-induced-phosphoprotein 1 for bovine: ALDLDSNCK, Hemoglobin subunit beta for sheep: FFEHFGDLSNADAVMNNPK	ND	**Wang et al., (2019b)**
Pork gelatin adulteration in meat products	High-resolution MS	Type I collagen: TGETGASGPPGFAGEK, HGNRGEPGPAGSVGPAGAVGPR	0.1% (w/w) of undesired pork gelatin	**Yang et al., (2018)**
Buffalo, sheep, and goat meat in minced meat and meat products	MALDI-TOF MS	Myosin light chain 1 for sheep: EAFLLYDR, Myosin light chain 2 for buffalo: NMWAAFPPDVGGNVDYK, Myosin light chain 1 for goat: EAFLLYDR	1.0% for raw meat and 0.1% cooked samples	**Naveena et al. (2017)**
Chicken blood in sheep whole blood samples	Internal extractive electrospray ionization mass spectrometry (iEESI-MS)	Hemoglobin for blood samples, peptide marker Not determined	2% chicken blood in sheep blood	**Song et al. (2017)**
Water buffalo and sheep meat in raw and cooked ground meat mixtures	MALDI-TOF MS UPLC-QTOF	Myosin light chain 1 for sheep: EAFLLYDRTGDGK, Myosin light chain 2 for sheep: FSQEEIR; Myosin light chain 1 for sheep: EAFLLFDRTGECK, Myosin light chain 2 for sheep: FSKEEIK	0.5% (w/w) of buffalo meat in sheep meat	**Naveena et al. (2017)**
Beef and pork meat is highly processed food matrices	HPLC/ESI-MS/MS	Collagen a2-chain for beef: IGQpGAVGPAGIR, Collagen a2-chain for pork: TGQpGAVGPAGIR	2% pork meat in Bolognese sauce	**Prandi et al. (2017)**
Chicken, duck, and goose meat in processed meat products	Nano-LC-QTOF-MS/MS	Pyruvate kinase for chicken: EPADAMAAGAVEASFK, Alpha-enolase for duck: NYPVVSIEDPFDQDDWGAWK, Hemoglobin alpha-A for the goose: TYFPHFDLQHGSAQIK	1% (w/w) of chicken or pork in chicken, duck, and goose meat mixture, 0.8% (w/w) beef proteins in commercial poultry frankfurters	**Fornal and Montowska (2019)**
Meat adulteration in mammalian meat samples	Q Exactive Orbitrap LC-MS/MS	Myoglobin for pork: HPGDFGADAQGAMSK, Myosin-1 for horse: TLALLFSGPASADAEAGGK, Myosin-2 for beef: TLAFLFSGTPTGDSEASGGTK, β-Hemoglobin for lamb: FFEHFGDLSNADAVMNNPK, β-Hemoglobin for chicken: FFASFGNLSSPTAILGNPMVR	1% (w/w) of pork or horse meat in a mixture before and after cooking	**Orduna et al. (2017)**

Since the amino acid sequence of peptides is more stable than DNA during meat processing, they have an incomparable advantage in meat adulteration identification, especially for highly processed meat products and similar meat species **(Prandi et al., 2017)**.

CONCLUSION

Food adulteration occurs globally and in many facets and affects almost all food commodities. Adulteration not only constitutes a considerable economic problem but also may lead to serious health issues for consumers. Many of the methods for detection of food adulteration require elaborate steps of sample preparation before analysis involving high-end technologies and that makes the whole process difficult to perform and time-consuming. As the methods of adulterating foods have become more sophisticated, very efficient, and reliable techniques for the detection of fraudulent manipulations are required. The analytical techniques commonly used for meat and fish species identification can be broadly divided into protein-based and deoxyribonucleic acid (DNA)-based techniques. The protein-based methods include immunological assays, electrophoretic, and chromatographic techniques. These methods are fast and easy to perform and the investment in equipment is much less compared to DNA-based methods. Food chain transparency and full raw material traceability are primordial for an effective food fraud prevention system.

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INVESTIGATING CHEMICAL CHANGES DURING SNAKE FRUIT AND BLACK TEA KOMBUCHA FERMENTATION AND THE ASSOCIATED IMMUNOMODULATORY ACTIVITY IN *SALMONELLA TYPHI*-INFECTED MICE

Elok Zubaidah, Vania Valencia, Muhaimin Rifa'i, Ignatius Srianta, Ihab Tewfik

ABSTRACT

This study uncovered the chemical changes during kombucha's fermentation process and revealed the associated immunomodulatory activity in *Salmonella typhi*-infected mice. The snake fruit juice and black tea extract were processed into kombucha (a beverage known for its health benefits) by fermentation with SCOBY culture at room temperature for 14 days. Snake fruit kombucha showed high changes in fermentation parameters (total acidity, pH, and total sugar), as well as bioactive compounds and antioxidant activity. *Salmonella typhi* demonstrated a reduction in the population of CD8+TNFα+ and CD4+IFNγ+ of infected experimental animals. Both snake fruit kombucha and black tea kombucha have the potential to be utilized as an immunomodulator to circumvent unstable conditions of the immune system caused by *Salmonella typhi*. Black tea kombucha and snake fruit kombucha cancan raise the production of CD8+TNFα+ and CD4+IFNγ+ in mice infected with *Salmonella typhi*. In the group of normal mice, black tea and snake fruit kombucha were able to lower down the production of CD8+TNFα+, which is a potent mechanism to modulate the immune system. Further research is required to highlight the mechanism and role of black tea kombucha and snake fruit kombucha in the immune response that modulates and treats infection by *Salmonella typhi*.

Keywords: kombucha; snake fruit; black tea; immunomodulator; *Salmonella typhi*

INTRODUCTION

Snake fruit (*Salacca zalacca* (Gaerth.) Voss) is a popular tropical fruit in South East Asian countries. In addition to its appetizing taste, snake fruit provides many health benefits due to its sugar content, dietary fiber, selected vitamins and minerals, and antioxidant compounds **(Aralas, Mohamed and Abu Bakar, 2009; Suica-Bunghez et al., 2016)**. In our previous studies, we demonstrated that snake fruits have the potential to be processed into Kombucha **(Zubaidah et al., 2018a)**.

Kombucha is a fermented tea beverage, black tea is commonly used which is fermented by a symbiotic culture of bacteria and yeast (SCOBY) **(Jayabalan et al., 2014)**. Kombucha has shown several beneficial effects, such as inhibit pathogenic bacteria growth **(Sreeramulu, Zhu and Knol, 2000)**, acts as an antioxidant, protects hepar, in addition to its anti-cancer property **(Dufresne and Farnworth, 2000)**. Furthermore, it reduces inflammation severity, prevents arthritis, and enhances the immune system as an 'immunomodulator' **(Jayabalan et al., 2014)**. An immunomodulator is a compound that can modulate the immune system, which is needed to overcome the unstable condition of health complications caused by antigen. Clinically, immunomodulation mechanisms are categorized as immunoadjuvant, immunostimulant, and immunosuppressant. On the other hand, instability in the immune system caused by bacterial invasion increases the occurrence of serious disease, e.g. typhoid **(Abbas, Lichtman and Pilai, 2007)**. *Salmonella typhi* is a pathogenic bacteria that causes typhoid fever – a serious health issue globally **(Crump, 2019; Thung et al., 2017)**. It spreads through non-hygienic consumption of water and food. The bacteria can invade gut mucosal through microfold cells and infects the area without resulting in any clinical symptoms. Lack of inflammation response caused a late treatment and worsened the condition of the patient **(Khan et al., 2012)**.

Studies reported that snake fruit kombucha can lower fasting blood glucose, increases superoxide dismutase, reduces malondialdehyde level, and promotes pancreatic beta-cell- regeneration in the hyperglycemic rat. Furthermore, snake fruit kombucha was proven to have a similarly significant effect compared to metformin in treating diabetic rats with a dosage of 5 $mL.kg^{-1}$ bodyweights per day given orally for 28 days. These positive effects of snake fruit kombucha known to be related with its chemical composition such as phenol, tannin, hexane, 1-methyl-2, 2-furancarboxaldehyde, glucopyranose, and caffeine, which are produced during the

fermentation process **(Zubaidah et al., 2018b; Zubaidah et al., 2018c)**.

These beneficial effects of snake fruit kombucha, which have been reported, lacked scientific evidence to ascertain its potential immunomodulatory effect. Thus, this study aimed to investigate the chemical changes during fermentation of kombucha and its immunomodulatory activity in *Salmonella typhi*-infected mice, which will be ascertained through the population of CD4+TNFα+, CD4+IFNγ+, CD8+TNFα+, and CD8+IFNγ+.

Scientific hypothesis

The fermentation affects the chemical characteristics of the kombucha. The kombucha administration raises the production of CD8+TNFα+ and CD4+IFNγ+ in mice infected with *Salmonella typhi*.

MATERIAL AND METHODOLOGY

Material

Snake fruit (*Suwaru salak* cultivar) was obtained from a local farmer in Malang, East Java, Indonesia. Black tea was purchased from the local market. SCOBY culture was bought from Wiki Kombucha, Bali, Indonesia. *Salmonella typhi* was obtained from a national culture collection.

Snake fruit kombucha and black tea kombucha preparation

Peeled snake fruit was separated from its seed, cut, and washed with distilled water. Snake fruit was juiced in a food processor with distilled water at a ratio of 1:1 (w:v), then filtered. The juice was added with 10% sucrose (w/v) and brought to boil. While black tea extract was prepared by eight grams of black tea immersed in 1 L of boiling water, added with 10% sucrose (w/v), and let sit for 15 minutes. The prepared snake fruit juice or black tea extract was poured aseptically into a sterilized glass container, cooled until it reached room temperature, and then inoculated with 10% SCOBY culture (v/v). The container was covered with a sterile cloth and let aside to undergo fermentation at room temperature for 14 days. The cellulose layer was aseptically separated and the solution was subjected to chemical and immunomodulatory activity evaluation.

Chemical Analysis

Total acidity, total sugar, total dissolved solids [TDS] was analyzed according to **AOAC (1995)**. pH was measured by using a pH meter. Total phenolic content was determined according to **Yang, Paulino and Janke-Stedronsky (2007)**. Total flavonoid content was evaluated according to **Atanassova, Georgieva and Ivancheva (2011)**. Antioxidant activity (DPPH scavenging activity) was analyzed according to **Pinsirodom, Rungcharoen and Liumminful (2010)**. All analyses were carried out on a day 0 and day 14 of the fermentation process to ascertain any changes in both black tea kombucha and snake fruit kombucha.

Immunomodulatory activity evaluation

Thirty female Balb-C mice aged 12 weeks were adapted for 7 days given food and water ad libitum, then randomly categorized into 6 groups: Normal (N, healthy group), N-BTK (normal + black tea kombucha), N-SFK (normal + snake fruit kombucha), Infected with *Salmonella typhi* (I), I-BTK (infected + black tea kombucha), and I-SFK (infected + snake fruit kombucha), with each group, consists of 5 mice. The experimental protocols and procedures of care and use of animals used in the present study were approved by the Ethics Committee (ethical clearance No. 1059-KEP-UB). The National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) was followed in this experiment. Kombucha was given orally as much as 0.007 $mL.g^{-1}$ body weight per day for 21 days. On day 22, *Salmonella typhi* infection was carried out intraperitoneally with a dosage of 0.1 mL per mice with a concentration of 10^8 cells per mL. On day 29, lymph organ was taken for flow cytometry analysis to assess the population of CD4+TNFα+, CD4+IFNγ+, CD8+TNFα+, and CD8+IFNγ+.

Statistical analysis

The chemical characteristics data were analyzed with ANOVA at a significance level of 0.05% with SPSS. Flow cytometry data were analyzed with BD cell quest ProTM and statistically analyzed with ANOVA at a significance level of 0.05% with SPSS. A significant result was furtherly analyzed with Tukey.

RESULTS AND DISCUSSION

Chemical changes during fermentation

The fermentation process is a metabolic process that triggers simultaneously changes to characteristics of the medium including its nutritional contents and antioxidant activity. Changes in both black tea kombucha and snake fruit kombucha are presented in Table 1. The increase in total acid at the end of fermentation is the result of the culture metabolism which converts sugar into organic acids, mainly acetic acid as the primary metabolite. Other acids were also produced during bacteria metabolisms such as acetic acid, gluconic acid, glucuronic acid, L-lactic acid, malic acid, tartaric acid, and citric acid.

Table 1 Changes in chemical characteristics of black tea kombucha and snake fruit kombucha during fermentation.

Parameter	Black Tea Kombucha		Snake Fruit Kombucha	
	Day 0	Day 14	Day 0	Day 14
Total Acid (%)	0.21 ±0.02*	0.42 ±0.08*	0.83 ±0.07*	1.11 ±0.01*
pH	5.06 ±0.05*	4.90 ±0.02*	4.01 ±0.01*	3.07 ±0.01*
Total Sugar (%)	10.99 ±0.01*	8.27 ±0.04*	13.00 ±0.11*	8.09 ±0.03*
TDS (°Brix)	13.79 ±0.01*	11.79 ±0.01*	14.45 ±0.01*	12.23 ±0.01*

Note: Data is the average of 3 replications ±*SD*. A notation of * shows significant different at each parameter in the same day at significant level of $p > 0.05$.

High total acid increment in Snake fruit kombucha was predicted and caused by native acid in salak such as ascorbic acid **(Jayabalan, Marimuthu and Swaminathan, 2007; Jayabalan et al., 2014; Malbasa et al., 2011; Supapvanich, Megia and Ding, 2011)**.

Higher accumulation of organic acids during fermentation is related to lower pH value owing to acid ability to release H+ and cause a drop in pH level. By the end of the fermentation process, total sugar and total dissolved solid levels in the medium were lower compared to their levels at the beginning of fermentation as sugar is considered the primary carbon source for microorganisms that facilitates metabolism during fermentation. The reduction of TDS might be also caused by sedimentation of protein, pectin, pigment, and minerals.

The fermentation process not only changed the chemical characteristics of a medium, but also its bioactive components such as phenolic content, flavonoid content, and antioxidant activity **(Jayabalan, Marimuthu and Swaminathan, 2007; Bhattacharya, Gachhui and Sil, 2013)**. Changes in bioactive characteristics of black tea kombucha and snake fruit kombucha are presented in Table 2. Kombucha fermentation has been known to produce several enzymes such as invertase, cellulase, and amylase that catalyzes the breakdown of the chain between phenolic and medium complex that contributed to the increase of phenolic content after fermentation. On the other hand, epicatechin in tea and salak is known to undergo isomerization and depletion form microbes cell during fermentation resulting in in an increase of total flavonoid by the end of fermentation **(Essawet et al., 2015; Jayabalan, Marimuthu and Swaminathan, 2007; Supapvanich, Megia and Ding, 2011; Apriyadi, 2017)**. The antioxidant activity also increased during fermentation as the phenolic and flavonoid contents increased.

Animal Observation

The effect of treatment on mice body weight was monitored and evaluated on day 0, 7, 14, 21, and 28 the data were presented in Table 3.

Weight gain was observed in the healthy group, black tea kombucha, and Snake fruit kombucha (Normal, N-BTK, N-SFK). On the contrary, *Salmonella typhi* infection has led to

Table 2 Changes in bioactive characteristics of black tea kombucha and snake fruit kombucha during fermentation.

Parameter	Black Tea Kombucha		Snake Fruit Kombucha	
	Day 0	Day 14	Day 0	Day 14
Phenolic content (mg.L^{-1} GAE)	181.18 ±0.98	407.14 ±1.43*	280.48 ±0.55	534.94 ±0.24*
Flavonoid content (mg.L^{-1} QE)	3388.03 ±58.93*	3916.34 ±31.70*	3762.81 ±12.22*	4618.82 ±85.84*
DPPH scavenging activity (%)	76.62 ±0.13*	80.92 ±0.11*	77.22 ±0.42*	83.90 ±0.19*

Note: Data is the average of 3 replications ±*SD*. A notation of * shows significant different at each parameter in the same day at significant level of p >0.05.

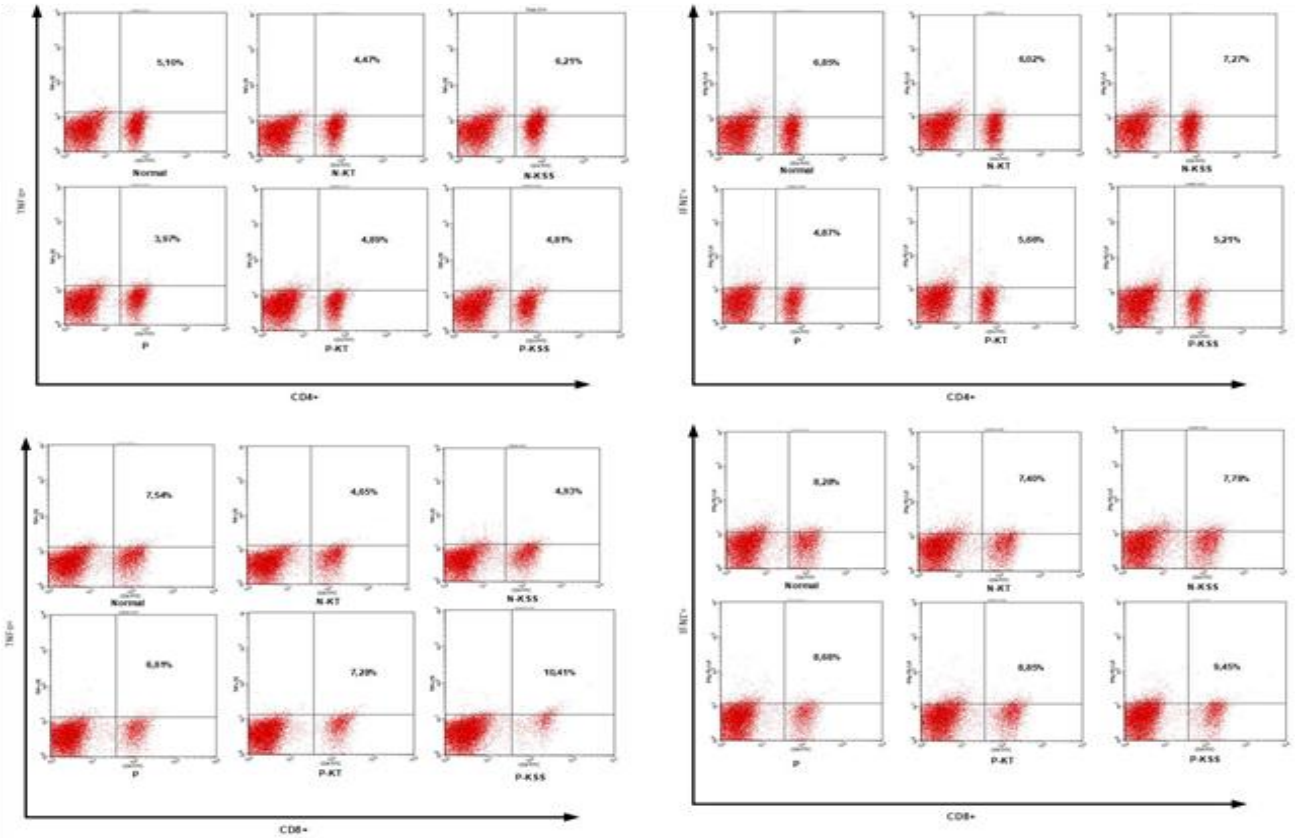

Figure 1 Flow cytometry analysis of CD4+TNFα+, CD8+TNFα+, CD4+IFNγ+, and CD8+IFNγ+.

Table 3 Changes in mice body weight during treatment (n = 5).

	Day 0	Day 7	Day 14	Day 21	Day 28
Normal	32.00 ±2.92	32.00 ±2.92	33.00 ±2.55	33.00 ±2.92	33.20 ±2.77
N-BTK	32.40 ±0.89	32.40 ±0.89	32.40 ±1.14	32.80 ±1.48	32.80 ±1.92
N-SFK	31.40 ±2.61	31.60 ±2.51	32.00 ±4.42	32.20 ±4.32	34.00 ±3.39
Infected	35.00 ±3.00	34.80 ±3.35	34.40 ±3.29	33.60 ±6.02	31.00 ±2.92
I-BTK	28.60 ±2.88	28.60 ±2.88	28.20 ±4.49	28.60 ±3.97	25.20 ±3.42
I-SFK	26.40 ±2.07	26.40 ±2.07	28.00 ±3.81	28.60 ±2.79	25.20 ±2.49

weight loss among an infected group, black tea kombucha, and snake fruit kombucha (Infected, I-BTK, I-SFK). Weight gain in the healthy group was related to the efficiency of gut activity in absorbing nutrients.

Moreover, high phenol, flavonoid, and antioxidant activity enhanced the body's metabolism to positive energy balance **(Fuller, 1989)**, thus healthy mice treated with snake fruit kombucha noted the highest weight gain (34.00 g).

Weight loss was a clear indication of *Salmonella typhi* infection. *Salmonella typhi* invade the gut mucosal surface and impaired the gastrointestinal tract absorption activity causing diarrhea, nausea, and vomit. The bacteria have also produced enterotoxin which stimulates gut epithelium to metabolize adenyl cyclase enzyme and c-adenosine monophosphate, which facilitated the secretion of chloride, natrium, and water from the gut lumen into the cell. In response to such conditions, hyperperistaltic occurred reduce excess water in the intestine thus diarrhea case has been established **(Ukhrowi, 2011; Nurhalimah, Wijayanti and Widyaningsih, 2015)**. Phenolic and flavonoid are known to have a bactericidal activity which is important to minimize the severity of diarrhea through inhibiting the growth of pathogenic bacteria **(Damayanti and Suparjana, 2007; Clinton, 2009; Loresta, Murwani and Trisunuwati, 2012)**. Acetic acid as the result of kombucha fermentation also correlates with inhibition of *Salmonella typhi* growth thus increases the efficiency of nutrient absorption, leading to weight gain **(Sreeramulu, Zhu and Knol, 2000)**.

Immunomodulatory effect of Black Tea Kombucha and Snake Fruit Kombucha

Figure 1 demonstrated the relative percentage of CD4+TNFα+, CD8+TNFα+, CD4+IFNγ+, and CD8+IFNγ+. Statistical tests noted that both *Salmonella typhi* infection and kombucha treatment did not reveal a significant effect on the relative percentage of CD4+TNFα+ and CD8+IFNγ+.

TNFα is an important cytokine produced in response to acute inflammation response stimulated by lipopolysaccharide. TNFα is needed to reduce pathogenic bacteria infection by inhibiting cell replication and destroying the infected cell. In the case of *Salmonella typhi* infection, TNFα+ mainly produced by CD8+ **(Oppenheim and Ruscetti, 2003; Bhuiyan et al., 2014)**. **Buttler and Girard (1993)** reported that TNF, IL-1, and IL-6 were increased as the response to *Salmonella typhi* infection. But, in this study we noticed that TNFα producing CD8+ has decreased. It is predicted that 3 – 7 days post-infection, macrophage effectively kill *Salmonella typhi* and eliminate dead cell **(Keuter, 1998)**, thus the expression of CD8+TNFα+ were lower (for instance, in the infected group), thus it can be inferred that both kombuchas have immunostimulant activity toward CD8+TNFα+.

Also, we revealed an immunosuppressant activity by both black tea kombucha and snake fruit kombucha. On the other hand, the non-infected group of mice treated with kombucha showed a lower CD8+TNFα+ relative percentage than the normal group. This may be due to bioactive components like flavonoid that causes lower expression of NF-kB transcription, followed by lower pro-inflammation cytokine production such as IL-17, IFNγ, and TNFα **(Saini, Sivanesan and Keum, 2016)**.

IFNγ is mainly produced by T- lymphocyte cells (CD4+ and CD8+) and natural killer cells which are activated as a response to antigen. High production of IFNγ increased the efficiency of macrophage to scavenge and kill microbes, initiate Th1 development, increase natural killer cells activity to lyse infected cell, increase MHC I expression which is needed by CD8+ to identify antigen, and increase MHC II expression to enhance the antibacterial activity **(Oppenheim and Ruscetti, 2003; Samuel, 2001)**.

Several immunological studies noted an increased level of IFNγ especially by CD4+ cells as a response to *Salmonella typhi* infection **(Sheikh et al., 2011)**. In this study, the population of IFNγ was decreasing compared to the normal group. Both black tea kombucha and snake fruit kombucha reported raising the population of CD4+IFNγ+ in which can be an alternative way to overcome the infection of *Salmonella typhi* since increasing IFNγ+ correlates to increasing activity of macrophage **(Abbas, Lichtman and Pilai, 2007)**.

Flavonoids also are known to have the ability to induce secretion of cytokines related to CD4+ cells and modulate its regulation by IL-2 production and increase CD8+ production **(Lyu and Park, 2005)**. Moreover, IL-2 is known to trigger CD8+ activation to produce perforin and grazin to support CD8+ function in destroying infected cells and *Salmonella typhi* antigen. Flavonoids are recognized to have immunostimulant activity by affecting macrophage and T cell and eliminate an infection. Flavonoids were able to activate the natural killer cell to trigger the production of IFNγ and increase the phagocytosis activity of macrophage. Also, phenols were proclaimed to initiate the production of IL-12 which activate natural killer cells to produce IFNγ and furtherly activate macrophage to kill antigen through the mechanism of oxygen-dependent- and oxygen-independent- **(Abbas, Lichtman and Pilai, 2007; Amit et al., 2017; Sulistiani and Rahayuningsih, 2015; Ramadhan, Mahfudh and Sulistyani, 2020)**.

CONCLUSION

Snake fruit kombucha triggers higher changes in chemical parameters during the fermentation process when compared to black tea kombucha. Moreover, snake fruit kombucha has higher bioactive components at the end of fermentation compared to black tea kombucha. Both products have the potential to be utilized as an immunomodulator to circumvent the unstable conditions of the immune system caused by *Salmonella typhi*.

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DIVERSITY OF WINTER COMMON WHEAT VARIETIES FOR RESISTANCE TO LEAF RUST CREATED IN THE V. M. REMESLO MYRONIVKA INSTITUTE OF WHEAT

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ABSTRACT

The results of the investigations of resistance winter common wheat varieties to leaf rust are given. The high resistance to the pathogen manifest varieties that contain resistance genes: *Lr9*, *Lr19*, *Lr37*, *Lr42* + *Lr24*, *Lr43* (*Lr21* + *Lr39*) + *Lr24*, *Lr9* + *Lr26*, *Lr10* + *Lr24* are ascertained. The genes *Lr13*, *Lr34*, *Lr37* in combination with other resistance genes provides long-term protection to leaf rust wheat. Winter wheat varieties, created at the V. M. Remeslo Myronivka Institute of Wheat, contain resistance genes *Lr23*, *Lr24*, *Lr26*, *Lr34*. The varieties Vesta, Snizhana, Demetra are protected by the resistance genes *Lr26* + *Lr34*, variety Zolotokolosa– *Lr24* + *Lr34*, Ekonomka – *Lr3* + *Lr26*, Myronivska storichna – *Lr3* + *Lr23* + *Lr10* + *Lr26*. The allele *Lr34* (+) is contained in varieties: Kryzhynka, Vesta, Snizhana, Volodarka, Demetra, Vdiachna, Pamiati Remesla, Sviatkova, Podolianka, Berehynia myronivska, MIP Dniprianka, and Balada myronivska. Sustainability is an important element of an integrated system of plant protection against many diseases, and to ensure increased yields it is necessary to create and distribute sustainable varieties that will be an environmentally promising way to develop the agro-industrial complex of Ukraine.

Keywords: common winter wheat; breeding; variety; resistance; rust; genes

INTRODUCTION

The leaf rust (*Puccinia recondita f.* sp. *tritici* Rob. Ex Desm.) is one of the most common and harmful wheat diseases. The disease leads to significant losses of grain yield **(Novohatka, 1979)**. The level of wheat yields loss to 30% during the epiphytotic rust according to the Food and Agriculture Organization of the United Nations (FAO) **(El-Khoury, 2009)**. The population of the pathogen *Puccinia recondita* differs from the high adaptive ability. High variability virulence of the fungus leads to the accumulation of pathogens that capable of the genes of wheat resistance **(Palamarchuk et al., 2019)**.

The most justified, economically sound, and environmentally safe method of fighting to disease is to creating resistance varieties. The effectiveness of breeding to rust resistance can be improved by using different Lr – genes **(Zheplinska et al., 2019)**.

More than 90 resistance genes to the rust-leaf pathogen are identified and characterized by chromosomal localization and efficiency at the wheat genome and its relatives **(Kovalyshyna and Dmytrenko, 2017)**, which information is collected annually and is published in the Catalogue of gene symbols for wheat **(McIntosh et al., 2003a; McIntosh et al., 2007b; McIntosh et al., 2008c; McIntosh et al., 2009d; McIntosh et al., 2010e; McIntosh et al., 2011f; McIntosh et al., 2012g; McIntosh et al., 2014h; McIntosh et al., 2016i; McIntosh et al., 2017j; McIntosh et al., 2018k; McIntosh et al., 2019l)**. Most genes of resistance to rust causative agents identified in cultivars of wheat descend from their wild relatives. Thus, according to the information provided in the catalogs of genetic symbols, about half of the known resistance genes to leaf rust are allogenic, introgressive into *Triticum aestivum* species from different types of wheat, aegilops, wheat grass, and others **(Leonova et al., 2013)**. The almost all effective rust resistance genes at the territory of Ukraine, except *Lr10* and *Lr23*, are allogenic, transferred to *Triticum aestivum* from other species: *Aegilops speltoides* – *Lr28, Lr35, Lr36, Lr47, Lr51, Lr66; Aegilops tauschii* – *Lr1, Lr21, Lr22a, Lr32, Lr39, Lr42; Triticum timopheevii* – *Lr18, Lr50; Thinopyrum elongatum* – *Lr19, Lr29, Lr24; Secale cereale* – *Lr25, Lr26, Lr45; Aegilops umbellulata* – *Lr19, Lr76; Triticum spelta* – *Lr44, Lr65, Lr71; Triticum dicoccoides* – *Lr53, Lr64; Aegilops triuncialis* – *Lr58, LrTr; Triticum timopheevii* spp. *viticulosum* – *LrTt1; Aegilops ventricosa* – *Lr37; Aegilops kotschyi* – *Lr54; Elymus trachycaulis* – *Lr55; Aegilops sharonensis* – *Lr56;*

Aegilops geniculate – Lr57; Aegilops peregrine – Lr59; Triticum turgidum – Lr61; Aegilops neglecta – Lr62; Triticum monococcum – Lr63. Therefore, in breeding must be taken into account the fact that in the wheat genome the effective *Lr*-genes are introduced not in its "pure" form, but closely linked with other genes that are undesirable to use in breeding **(Dinh et al., 2020; Lodgering et al., 1970)**.

It is a rather difficult task creating such varieties, and over time, they lose their resistance by the emergence of new races, pathogenic strains, and climatic changes. Trends in climate warming affect on the deterioration of the phytosanitary condition of crops **(Mushtruk et al., 2020)**.

Scientific hypothesis

The scientific hypothesis is founded on identifying nature inheritance and manifest resistance genes to exciter of leaf rust. It is attaining by investigation of composition population exciter of disease and identifies resistance genes at collectible samples soft wheat. It is making it possible to increase the resistance gene pool and creating new heterogeneous varieties of soft wheat.

MATERIAL AND METHODOLOGY

The assessment varieties (Table 1) of winter wheat were created at the V. M. Remeslo Myronivka Institute of Wheat for resistance to leaf rust made in conditions of the artificial infectious background of the wheat pathogen. Experiments were in the field conditions at a field infectious nursery of the Plant Protection Department of the V. M. Remeslo Myronivka Institute of Wheat. The climate is temperate continental. The average annual air temperature is 7.6 °C. The sum of effective temperatures above 5 °C is 3000 °C. The duration of the frost-free period becomes an average of 165 days. The average annual amount of decline is 310 – 570 mm.

A suspension of a mixture of spores isolated from the local leaf rust population was used for inoculation. Wheat varieties were inoculated a mixture of spores with talc in the ratio of 1:100 by a technique of **Geshele (1971)** in a tubing phase – beginning form ears in condition artificial infectious nursery. The spore load was 0.015 kg of urediniospores per one m^2 of sowing. The assessment of resistance was conducted dynamically every 10 days **(Tkachyk, 2014)**. The variety Myronivska 10 was used as a susceptible standard. The accounts defeat by the causative agent were evaluated on the scale according to **Trybel et al. (2010)** and **Bober et al. (2020)**. A DNA was isolated from weight 25 – 40 mg of the plant material, obtained by grinding of 5 grains in ceramic mortars to a homogeneous powder and the further selection and weighing **(Babaiants, 2011)**. DNA isolation was conducted using sets of Diatom™ DNA Prep100 (NEOGENE, Ukraine) according to a standard procedure with certain modifications **(Trybel et al., 2010)**.

The caSNP12 marker was used in investigations of genetic material for identification allelic status of the resistance gene to wheat leaf rust Lr34, **(Radchenko and Odintsova, 2008)**. PCR was performed on the amplifier GeneAmp® PCR System 2720 (Thermo Fisher Scientific, Massachusetts, USA) using sets GenPak® PCR Core according to the manufacturer's method. The primer sequences are as follows: for caSNP12F – 5'-TCCCCAGTTTAACCATCCTG-3'; for caSNP12R – 5'-CATTCAGTCACCTCGCAGC-3' (NEOGENE, Ukraine).

The conditions of the PCR were in line with the requirement recommended by the developer. As a result of PCR with a mixture of primers flanking the caSNP12 marker **(Strahov, 1951)**. The stable allelic state of the markers (- Lr34 +) answered amplicons with a length of 234 p. n., the sensitivity was the absence of amplicons **(Vyerchenko et al., 2019)**.

Fragments received from PCR were separated in 1.8% agarose gel. Ethyl bromide was used as an intercalating agent for DNA monitoring in an ultraviolet. The system VISION Gel (Scie-plas, Great Britain) was used for gel documentation. The length of the clearest and reproducible bands was determined using DNA marker O'Gene Ruler 50 bp Plus DNA Ladder (Fermentas, Lithuania).

Statistical analysis

To obtain information on the number and interaction of resistance genes, the obtained ratios of classes of resistant and susceptible plants (actual) were compared with one of the theoretically expected cleavages using the chi-square (χ^2) correspondence criterion. The assumption that the difference between the actually obtained and theoretically expected splits is random was rejected if $\chi^2_{fact.}$ exceeded the critical $\chi^2_{st.}$ ($\chi^2_{0.05}$ = 3.84). An error of results in statistical analysis p = 0.05. Statistical processing was performed in Microsoft Excel 2016 using the analytical application XLSTAT from Addinsoft for Microsoft Excel. Values were estimated using mean and standard deviations.

RESULTS AND DISCUSSION

The genes of high efficiency against the pathogen are *Lr9, Lr19, Lr37, Lr42+Lr24, Lr43 (Lr21+Lr39) +Lr24, Lr9+L26, Lr10+Lr24* discovered by investigation the population of leaf rust on varieties of carriers of effective resistance genes **(Leary et al., 2018; Ramanathan et al., 2018)**. The resistance of the varieties protected by the Lr24 genome is lost. We observed a slight defeat by the pathogen of the carriers of the Lr19 gene, indicating that the population has virulent clones against it. The pustules of leaf rust were marked also on varieties protected by the genome Lr9 in some years. The genes *Lr37*, *Lr42 + Lr24* and *Lr43* (*Lr21 + Lr39*) + *Lr24* have high efficiency in recent years. The *Lr13*, *Lr34*, *Lr37* genes provide the long-term protection of wheat against rust in combination with other genes **(Dorofee, 1972; Bharti et al., 2016)**. The varieties with these genes remain resistant for 20 – 30 years in different countries of the world **(Leppik, 1970)**.

10 highly resistant endemic species of wheat and its relatives with the highest immunity to rust, in particular: *Triticum monococcum*, *T. timopheevii*, *T. militinae*, *T. Zhykovski*, *T. fungicidum*, *Haynatriticum*, *Aegilops umbellulata* are distinguished **(Varella et al., 2017; Jafary, Szabo and Niks, 2006)**.

Table 1 Genetic diversity of winter wheat varieties for resistance to leaf rust.

Variety	Owner of Variety	Entry into the State Register, year	*Lr*- genes	Plant damage, %, the average for 2015 – 2018 pp.	Resistance, number
Ukrainka 0246	MIW[1]	1929	*Lr16*	13.8	6
Myronivska 264	MIW	1960	*Lr3, Lr16*	9.5	7
Myronivska 808	MIW	1963	*Lr3*	17.5	5
Myronivska 61	MIW	1989	*Lr26*	11.3	6-7
Myronivska 27	MIW	1992	*Lr26*	8.3	7
Myrleben	MIW	1993	*Lr26*	18.3	5
Myronivska 28	MIW	1994	*Lr26*	4.5	8
Myronivska 30	MIW	1995	*Lr26*	18.8	5
Myronivska 31	MIW	1997	*Lr26*	7.5	7
Myronivska 33	MIW	1998	*Lr26*	11.3	6-7
Myrych	MIW	1999	*Lr26*	10.0	7
Myronivska 65	MIW	2000	*Lr26*	16.3	6
Kryzhynka	MIW	2002	*Lr26, Lr34*	8.3	7
Myronivska 67	MIW	2002	*Lr26*	15.0	6-7
Kolumbiia	IPPG[2], MIW	2003	*Lr24*	2.8	8
Podolianka	IPPG, MIW	2003	*Lr34*	11.3	6-7
Snizhana	MIW, IPPG	2004	*Lr26, Lr34*	10.0	7
Pereiaslavka	IPPG, MIW	2004	*Lr26*	13.8	6
Smuhlianka	IPPG, MIW	2004	*Lr24*	4.5	8
Demetra	MIW, IPP[3]	2005	*Lr26, Lr34*	15.0	6-7
Vesnianka	IPPG, MIW	2005	*Lr24*	5.3	8
Volodarka	IPPG, MIW	2004	*Lr34*	5.0	8
Favorytka	IPPG, MIW	2005	*Lr26*	5.0	8
Pyvna	IPPG, MIW	2006	*Lr26*	12.5	6-7
Zolotokolosa	IPPG, MIW	2006	*Lr24, Lr34*	12.5	6-7
Kalynova	MIW, IPPG	2008	*Lr26*	20.8	5
Kolos Myronivschyny	MIW, IPPG	2008	*Lr26*	13.8	6-7
Monotyp	MIW, IPPG	2008	*Lr26*	7.5	7
Ekonomka	MIW, IPP	2008	*Lr3, Lr26*	5.0	8
Pamiati Remesla	MIW, IPPG	2009	*Lr34*	5.8	7
Myronivska storichna	MIW, IPP	2009	*Lr3, Lr10, Lr23, Lr26*	5.8	7
Yuviliar Myronivskyi	MIW, IPPG	2009		12.5	6-7
Myrliena	MIW, IPPG	2009	*Lr23*	5.8	7
Yasnohirka	IPPG, MIW	2009	*Lr24*	4.0	8
Slavna	IPPG, MIW		*Lr24*	8.9	7
Yavoryna	IPPG	2010	*Lr24*	10.0	7
Lehenda Myronivska	MIW	2012	*Lr26*	10.0	7
Myronivska (standard 10 of susceptibility)	MIW			26.3	3-4

Note: 1 MIW – the V. M. Remeslo Myronivka Institute of Wheat of the National Academy of Agrarian Sciences of Ukraine; 2 IPPG – Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine; 3 IPP – Institute of Plant Protection of the National Academy of Agrarian Sciences of Ukraine.

In the works **(Casey et al., 2016; Marryat, 1907; Davoyan et al., 2012)** found forms immune to rust, powdery mildew, and soot among *Triticum dicoccum*.

The principles of researching gene pool of wheat resistance against leaf rust, based on **(Flor, 1971)** theory of "gene-for-gene", developed **(Berlyand-Kozhevnikov et al., 1985)**.

In Ukraine, wheat breeding for rust resistance is conducted for more than 40 years. Scientific researches on the study of genetic signs of resistance to the causative agent of leaf rust actively unfolded in the late 70's – the early 80's of the twentieth century **(Morgounov et al., 2011)**. The greatest successes have been achieved at the V. M. Remeslo Myronivka Institute of Wheat of the National Academy of Agricultural Sciences of Ukraine, the Plant Breeding and Genetics Institute – National Center of Seed and Cultivar Investigation of the National Academy of Agricultural Sciences of Ukraine, the Institute of Plant Protection of National Academy of Agrarian Sciences of Ukraine and the Plant Production Institute nd. a. V. Ya. Yuryev of National Academy of Agrarian Sciences of Ukraine **(Khaneghah et al., 2018; Kirilenko, 2014)**.

We conducted researches on the detection of the resistance gene to leaf rust in winter wheat varieties of Myronivka's breeding. The *1BL/1RS* wheat-rye translocation, which carries the resistance gene to leaf rust Lr26, as well as resistance genes to stem rust Sr31, powdery mildew Pm8, yellow rust Yr9 were found in the following varieties: Myronivska 61, Myronivska 27, Myrleben, Myronivska 28, Myronivska 30, Myronivska 31, Myronivska 33, Myrych, Myronivska 65, Myronivska 66, Kryzhynka, Myronivska 67, Vesta, Snizhana,

Table 2 Allelic status of the *Lr34* gene in new winter wheat varieties of MIW by results of PCR on the marker *caSNP12*, 2017.

Variety	Entry into the State Register, year	Allelic status of the gene *Lr34**
Svitanok Myronivskyi	2014	-
Horlytsia myronivska	2016	-
Hospodynia myronivska	2017	-
Berehynia myronivska	2016	+
MIP Kniazhna	2017	-
MIP Vyshyvanka	2017	-
MIP Valensiia	2017	-
Myronivska slava	2017	-
Trudivnytsia myronivska	2017	-
Estafeta myronivska	2018	-
Vezha myronivska	2018	-
MIP Dniprianka	2018	+
Balada myronivska	2018	+
Hratsiia myronivska	2018	-
MIP Assol	2018	-

Note: * "+" – Associated with allele resistance *Lr34* (+); "-" – allele present in susceptible varieties Lr34 (-).

Pereiaslavka, Demetra, Favorytka, Pyvna, Kalynova, Kolos Myronivschyny, Monotyp, Ekonomka, Myronivska storichna, Lehenda Myronivska according to the results of investigations by **Kovalyshyna et al. (2020)**, **Lisova (2012)** and **You-Xiong et al. (2009)**. The *Lr26* gene does not provide high resistance of varieties to leaf rust causative agent, since this gene has lost its effectiveness against the population of the disease as the data in Table 1 show.

The availability of wheat-rye translocation *1AL/1RS* in the genotype of winter wheat provides resistance to fungal diseases, because it carries a complex of resistance genes – to leaf rust *Lr24*, stem rust *Sr1RS*, powdery mildew *Pm17*. This translocation was detected in varieties: Kolumbiia, Smuhlianka, Vesnianka, Zolotokolosa, Yasnohirka, Slavna, Yavoryna **(Vlasenko, Koliuchyi and Chebakov, 2005; Landjeva et al., 2006)**. The *Lr24* gene provides moderate resistance to leaf rust, varieties have damage of 2.8 – 17.2%.

The varieties Vesta, Snizhana, Demetra are protected by the *Lr26* and *Lr34* genes, and the Zolotokolosa – by the *Lr24* and *Lr34*. The combination of these resistance genes inhibits the development of leaf rust on winter wheat varieties, damage of the leaf surface is within 10 – 17.5%

The Myrliena variety contains the gene *Lr23*, and the Myronivska storichna – *Lr3, Lr23, Lr10,* and *Lr26* are ascertaining according to the analysis of the genealogy of varieties. The Lr3, Lr24 genes, protects the Ekonomka variety.

The gene *Lr34*, which belongs to the group of genes that provide partial resistance in the phase of adult plants, is found in the following varieties: Kryzhynka, Vesta, Snizhana, Volodarka, Demetra, Vdiachna, Pamiati Remesla, and Sviatkova **(Kozub et al., 2017; Pirko et al., 2012)**. Eight varieties exhibit polymorphism for the gen *Lr34* – Illichivka, Myronivska 30, Myronivska 32, Myronivska 65, Myronivska 66, Pyvna, Ekonomka, Lehenda Myronivska. Perhaps a small frequency of the dominant allele of the *Lr34* gene in the varieties of MIW is related to the difficulty of identifying it in field conditions in the Right-Bank Forest-Steppe zone of Ukraine with the use of classical breeding methods, since for varieties of the Plant Breeding and Genetics Institute – National Center of Seed and Cultivar Investigation of the National Academy of Agricultural Sciences of Ukraine frequency is much higher. The varieties Zolotokolosa and Podolianka also contain the gene *Lr34* according to **Radchenko and Tishchenko (2010)** and **Krattinger et al. (2016)**.

We found that among the 15 newly created varieties, only 3 varieties – Berehynia myronivska, MIP Dniprianka, and Balada myronivska contain the *Lr34* allele (+), which is only 20% of the investigated varieties according to the PCR results, using the *caSNP12* marker (Table 2).

CONCLUSION

It has been established that effective against the pathogen of leaf rust the are genes: *Lr9, Lr19, Lr37, Lr42+Lr24, Lr43 (Lr21+Lr39) +Lr24, Lr9+L26, Lr10+Lr24*. The genes Lr13, Lr34, Lr37 in combination with other genes, provide the long-term protection of wheat against leaf rust.

The wheat-rye translocation *1BL/1RS* was identified in the following varieties: Myronivska 61, Myronivska 27, Mirleben, Myronivska 28, Myronivska 30, Myronivska 31, Myronivska 33, Myrych, Myronivska 65, Myronivska 66, Kryzhynka, Myronivska 67, Vesta, Snizhana, Pereiaslavka, Demetra, Favorytka, Pyvna, Kalynova, Kolos Myronivschyny, Monotyp, Ekonomka, Myronivska storichna, Lehenda Myronivska.

The availability of wheat-rye translocation *1AL/1RS* was found in varieties: Kolumbiia, Smuhlianka, Vesnianka, Zolotokolosa, Yasnohirka, Slavna, Yavoryna.

The *Lr34 (+)* allele is presented in varieties: Kryzhynka, Vesta, Snizhana, Volodarka, Demetra, Vdiachna, Pamiati Remesla, Sviatkova, Podolianka, Zolotokolosa, Berehynia myronivska, MIP Dniprianka, and Balada myronivska according to the results of the research. As a result of research, it was found that the wheat variety Myronivska Ukrainian selection is quite productive in comparison with other varieties and resistant to pathogens of various diseases, especially to the pathogen (brown rust).

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SUBSTANTIATION OF FOAMY STRUCTURE FORMATION IN A GLUTEN-FREE BISCUIT

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ABSTRACT

A promising way to create gluten-free foods based on purposeful use of ingredients with a wide range of technological properties is analyzed. Steps to regulate the structural and mechanical properties of gluten-free dough have been determined. These steps allow to improve the structural-mechanical and organoleptic characteristics of the biscuit semi-finished product, to adjust the nutritional value. To determine the technological effect, we considered the connection between the recipe components and the properties of the dough when forming a foamy structure of gluten-free cupcake products. The influence of the design parameters of the mixer (independent factors xi) on the foaming process (Q) has been determined, that is, determining the productivity magnitude from the changes of three main factors: from the attack angle of the frontal surface of the plate working body α, the distance between the plates (step) t and the rotation frequency of working body n. The stability of the foam formed and the rate of its settling and the role of the liquid phase of the dough during short storage before baking were investigated. The comparative characteristics of the microstructure of wheat flour (WF) and extruded corn flour (ECF) in the ratios are presented: a) WF – 100 wt.%; b) WF: ECF – 80:20 wt.%; c) ECF – 100 wt.%. In the tested mixtures, the moisture-holding capacity increases for the sample containing 20% by weight of extruded corn flour two and a half times, and three times for the sample with extruded flour to 100% by weight. With an increasing proportion of extruded corn flour in the flour mixture, the dough density increases and the optimum value is in the range of 0.444 – 0.446 kg.m^{-3}. The comparative characteristic of the microstructure of the samples is given, which has the appearance of foam with the existing and even distribution of air bubbles which later form the porous structure of the biscuit of the semi-finished product. Thus, the size of the formed bubbles of air with the content of wheat flour and starch have a large difference in diameters, in the sample of biscuit dough using ECF 100 wt.% – almost the same size, and between the channels are formed that promote the equalization of air pressure in the middle of the foam system of biscuit dough. It was found that the use of 20 wt.% and 100 wt.% of corn extruded flour contributes to the formation of a fine porous structure of biscuit dough.

Keywords: dough; adhesion; adhesive; substrate; forming channel; deformation; stress

INTRODUCTION

Today, the popularity of healthy eating is increasing every year. This requires the creation of products that contain ingredients with wellness properties. The tissues of the human body include mineral compounds that make up about 3.5% of its mass. Therefore, when consuming food, they should contain the required amount of salt, phosphorus, calcium, iron, etc. The daily requirement of the human body in trace elements is measured in milligrams (zinc: 10 – 15, manganese: 5 – 10, copper: 2, molybdenum :0.5). Their physiological value is quite significant, as these trace elements play the role of coenzymes of some enzyme systems.

An analysis of the literature shows that the share of gluten-free food on the Ukrainian market is quite small, and the percentage of people who go to health care establishments with increased sensitivity to gluten is increasing, even among children. Also, there is increasing public awareness of gluten intolerance, that is why people choose gluten-free foods as an attribute of healthy eating. In western countries, the consumption of gluten-free products is becoming a lifestyle, which contributes to increasing their production. Thus, according to experts, in the UK in 2019, the market for gluten-free products reached 1 billion dollars. In Ukraine, however, the industrial production of gluten-free products is not established, but to provide the category of people with coeliac disease, specialized food needs constantly.

Considering the directions of technological process in the food industry **(Lamacchia et al., 2014)**, determined in particular by public policies on healthy nutrition, economic and social change in society, new technological opportunities, and competition in the food market, there is a need not only for the improvement of traditional food technology but for the creation of new generation foods which are enriched with important nutrients and have

a longer shelf life. Also, an important role is played by equipment, which creates the appropriate conditions for modern technological requirements. Semi-finished flour confectionery products form the basis or constituent of products **(Murray, 2007)**, and significant demand from the population for these products makes them considered important food products.

Today, in particular **(Liu, Willför, and Xu, 2015; Kobets et al., 2015)**, the range of gluten-free food produced in domestic production is insufficient. The range of gluten-free flour products on the Ukrainian market is formed mainly due to imported products, which have a fairly high cost. To solve the problem of nutrition for patients with coeliac disease in Ukraine products of the firm "DR. SCHAR" (Italy), "BEZGLUTEN" (Poland), "3PAULY" (Germany) are certified but their use is limited because of the high price. The range of food products for people suffering from genetically caused and allergic diseases in our country is not wide enough and is about 2%. This indicates that the issue of developing special-purpose technology products, including for the nutrition of people with coeliac disease, in Ukraine is quite acute and relevant. Therefore, one of the promising directions of expanding the range of flour confectionery products is the creation of biscuit semi-finished products, bagels with the development of structural and technological conditions of the process with the complete replacement of gluten-containing wheat flour with extruded corn flour (ECF).

ANALYSIS OF THE LATEST RESEARCH

In the general structure of the market of flour confectionery products (FCP) cupcakes and bagels occupy up to 15% of the total volume of production. These products have a pleasant appearance and taste, are well absorbed by the human body and therefore are popular with the population. Analysis of the literature indicates that the use of gluten-free flour in the production of pastry, including muffins on chemical baking powder causes many technological problems and requires a variety of tools to improve the structure of the gluten-free dough. The fact is that gluten of wheat flour has unique technological properties that play an important part in the formation of flour dough structural and mechanical qualities and texture of finished products. Wheat Glyadine (Prolamine) is responsible for the dough's consistency as well as Gluten (Glutinine) is responsible for the resistance of the tensile test. The combination of these two proteins gives the dough unique viscoelastic properties and the ability to hold gas. After hydration and mixing, proteins of gluten-free flour varieties do not develop into a viscoelastic network **(Kobets et al., 2015)** as wheat proteins.

During the development of the cupcakes, it was found that simply replacing the wheat flour with rice does not allow the product to have the necessary structural characteristics. It has been established **(Stojceska et al., 2010; Drobot, Pysarets and Kravchenko, 2013)** that reducing the amount of rice flour by 10% is sufficient to improve the structure of the cake. The product has a developed porosity and a high specific volume.

The possibility **(Rus'kina et al., 2017)** of using protein and protein-calcium concentrates from white and brown rice in the technology of gluten-free oil cake has been investigated. The obtained rice concentrates were characterized by high foaming capacity and foam stability, which allowed them to be used in the amount of 50% by weight of egg melt in oil cake technology to increase its biological value and structural and mechanical quality indicators.

The main problem of manufacturing gluten-free flour products is an imitation of the structure-forming properties of gluten-containing raw materials. Since the biscuit semi-finished product is a structured foam-emulsion dispersive system, the formation of the necessary rheological properties that provide a texture adequate to a traditional product is an important task for its creation. In the technological aspect, the solution of this problem is to find the optimal ratio of structure-forming components, the choice of design conditions for the formation of a stable structure of the food system, its structural and mechanical qualities, characterized by viscosity, elasticity, plasticity.

Corn flour starch **(Stojceska et al., 2010)** is pasteurized at higher temperatures, more easily attacked by amylase, contains less own sugars, less sugar-forming, and gas-forming ability.

Many studies by several scientists **(Stojceska et al., 2010; Drobot, Pysarets and Kravchenko, 2013)** have developed measures for the use of a mixture of wheat and corn flour while maintaining pre-mixing corn flour. They consist of soaking, brewing, fermenting with mesophilic or thermophilic lactic acid bacteria, adding unfermented malt, phosphates, concentrates, or enzymes to the dough. However, these measures extend the duration of the process and do not provide the significant effect of improving product quality at considerable cost. Recently **(Stojceska et al., 2010; Drobot, Pysarets and Kravchenko, 2013)** began to develop measures for the use of whey.

The availability of fundamental developments in the production and use of various types of extruded flour in food production **(Brekhov and Riazhskykh, 2012; Yehorov, Mardar and Bordun, 2014; Zip Diagnostic, 2012)** indicates the possibility of its use in biscuit technology and production of bagels. The use of plant-based ingredients, such as non-traditional flour types, which could provide valuable nutritional value to the product, may be considered a promising development of interest to confectioners and bakers.

Because of the valuable chemical composition of the ECF, it is possible to predict the positive impact of this raw material on the course of the technological process.

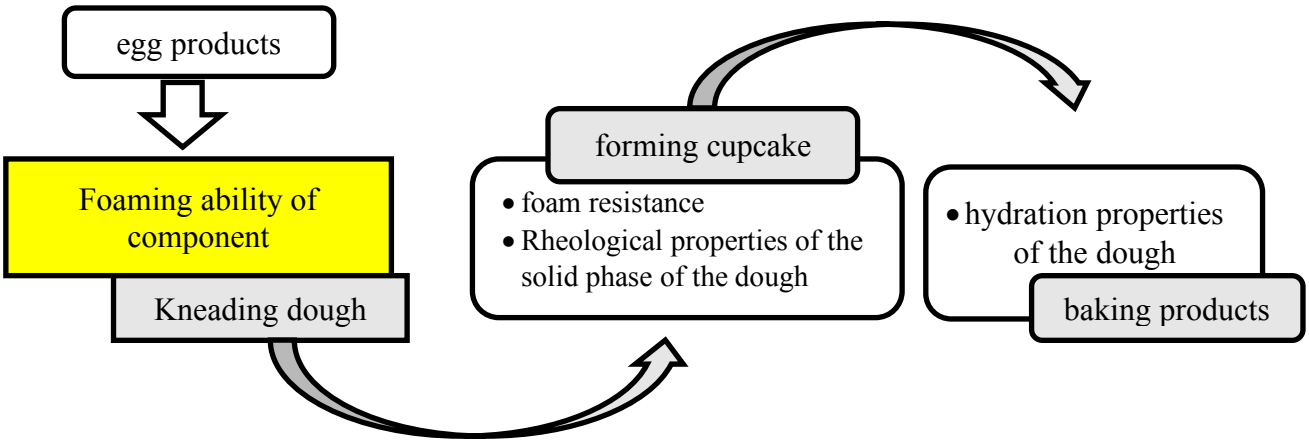

Figure 1 The relationship between recipe components and properties of the dough mass and the foamy structure formation.

In the technological process of mixing the biscuit semi-finished product as a foaming agent, egg mélange is most often used. Due to the saturation of the protein mass with air, the knockdown causes the denaturation of the protein's native structure, which in its turn unfolds the protein chains without destroying their covalent structure. In the process of denaturation, the protein's inherent three-dimensional structure breaks down **(Renzjaeva and Bakirova, 2017),** the polypeptide chain unfolds, simultaneously forming bonds between the polypeptide chains, which generally contributes to the stability of the foam. The foamy structure of the biscuit semi-finished products is due to a greater extent to the presence in their recipe of a considerable quantity of egg products that is almost twice more than flour (Figure 1). This makes it possible to assume that wheat flour will be completely replaced by non-baking flour.

Therefore, biscuit dough is a complex dispersed system consisting of air bubbles separated by liquid pellicle. In this system, the dispersed medium is an egg-sugar mixture and the dispersed phase is air. The technological process **(Drobot, Pysarets and Kravchenko, 2013)** involves dispersion of the egg-sugar mixture, which is saturated with air bubbles and leads to an increase in volume, development of the inner surface of the system, and creation of a foam system.

The main characteristics **(Renzjaeva and Bakirova, 2017; Drobot, Pysarets and Kravchenko, 2013)** that comprehensively characterize the foam system are – foaming solution, foam multiplicity, foam stability, and foam dispersion, i.e. distribution bubbles by the size or surface of the solution-gas separation, per unit volume of foam. The patterns that cause the formation of foam vary significantly depending on the conditions of mixing components.

The beating of the egg-sugar mass in ECF technology is one of the separate operations which results in the formation of a system that is a confectionery foam. The formation of thick foam is positively affected by the surface activity of egg polar white molecules and a noticeable stabilizing effect is present in the sugar mixture. As a result, solid pellicles are formed around the air bubbles which enhance the foam's stability. Increases the marked effect of increasing the viscosity of the liquid when sugar is added.

Along with the prescription components, the duration of the whipping and the speed of rotation of the working organs are influenced by the foam volume and its dispersion, the foaming ability, and the foam resistance. As the whipping time increases, the volume of foam also increases; the whipping rate is directly proportional to the foam dispersion.

Today, a considerable amount of theoretical and applied aspects of foam production **(Holovchenko, Lopatin and Kovbasa, 2001)**, physicochemical properties, structure characterizing the geometric shape of air bubbles and their stability have been accumulated. The structure of the foams is determined by the ratio of the volumes of liquid and gas phases and, depending on this ratio, the foam cells have a spherical or polyhedron shape. Usually, freshly prepared foam of the egg-sugar mixture has a spherical shape of cells separated by thick walls of liquid, because the volume of the gas phase exceeds the volume of the liquid by no more than 10 – 20 times. As the foam ages, the spherical shape of the foam bubbles becomes multifaceted with thin flat pellicles as a result of fluid draining. At the same time, the specific surface of the foam changes due to the diffusion of gas from small bubbles into larger ones, due to the difference in capillary pressures.

In the paper **(Holovchenko, Lopatin and Kovbasa, 2001)**, it is noted that although the foam state with polyhedral cells is close to equilibrium, such foam has greater stability than the one with spherical cells. However **(Holovchenko, Lopatin and Kovbasa, 2001; Mancebo, Rodriguez and Gómez, 2016)**, by reducing the number of small bubbles, the total number of air bubbles in this volume of foam decreases, that is, the coalescence of foam occurs, which reduces its stability. Carried out studies **(Mancebo, Rodriguez and Gómez, 2016)** show that with increasing foam concentration, the viscosity of the solution decreases and foaming improves, and the density of the foaming mass decreases due to more air being drawn into the system.

A characteristic feature of biscuit dough is that in its composition, in addition to foaming agents other recipe components are included that have a significant impact on the quality of the biscuit semi-finished product. The main

component that has a significant impact on the quality of the biscuit cake mix is flour.

Compared to the duration of the whisking of the egg-sugar mass, the kneading with the flour lasts only a few seconds, but the flour has a significant effect on the quality of the semi-finished product. The properties of gluten flour and its content significantly affect the quality of the baked semi-finished product, because when kneading occurs the hydration of gluten proteins and longer kneading leads to the tightening of the dough and compacting the structure of the biscuit semi-finished product.

The research **(Stojceska et al., 2010)** identified the steps of regulating the structural and mechanical properties of gluten-free dough. First of all, it is the use of flour mixtures, not certain types of gluten-free flour, which can significantly improve the nutritional and biological value as well as product structure but to expand the raw material base and finished product range. To establish the technological effect, we considered the relationship between the recipe components and the properties of the dough masses in the formation of the foamy structure of gluten-free cupcake products (Figure 1).

In physics and chemical sense, foam is a two-component gas-liquid system. In the process of foam formation, there is a powerful development of the liquid and gaseous phase interface. The surface tension force always seeks to minimize the total surface of the section. In the dough foam mass, the flour component actively absorbs the moisture from the liquid phase contained in the foam. This leads to the rupture of the pores pellicles, followed by their merging into pores of larger sizes. Hence there is the formation of heterogeneous porosity in the finished products prepared based on flour.

In the production of the cupcake, when the mixture turns into the dough, gluten forms a viscoelastic network capable of trapping and storing carbonated bubbles. The state of aeration of the dough immediately after mixing has a huge impact on the texture of the cupcake. And the aerated dough architecture is governed by different physical principles related to foam formation and stabilization.

The specificity of the production of gluten-free dough by beating is that the foam obtained is subjected to unwanted external influences that lead to a decrease in its stability. Such factors include mixing the whipped mixture with flour and placing the dough in molds (Figure 4). In such circumstances, it is important not only to obtain a foam system with the required characteristics but also to preserve them during the technological process. Thus, in our view, it is important to investigate the foaming ability and foam resistance to structure destruction comprehensively.

According to current scientific concepts **(Drobot, Pysarets and Kravchenko, 2001; Haliasnyi, Gavrish and Shanina, 2018)**, in the absence of a hydrated gluten network, one of the important factors for optimizing and stabilizing the process of retaining gas formed in the gluten-free dough is sufficient water amount to hydrate the biopolymers of the dough and obtain the desired viscosity. It is possible to increase the hydration capacity of gluten-free dough by adding protein substances.

For the formation of a stable foam structure, it is necessary to weaken the counteraction of the system surface tension which is achieved by introducing into the mass of the surfactants which can significantly reduce the surface tension at the interface. We have developed **(Stadnyk et al., 2019)** a method of attenuating the surface tension forces of a system by using a discretely pulsed mixing machine. Its purpose is to intensify the technological process by reducing the time of beating the egg-sugar mixture. It should be noted that obtaining a well-loosened dough structure is the first but not the only technological task. Also important is the preservation of the formed structure which determines the corresponding rheological properties of the dough.

Scientific hypothesis

The idea of the development is to establish the relationship of wheat and extruded corn flour (ECF) in the process of mixing the recipe components on the developed mixer with characteristic differences in the formation of a stable foam structure in the technology of cupcakes.

MATERIAL AND METHODOLOGY

Materials

Extruded Corn Flour (ECF) of maize crop (2018, 2019) was grown in Chernihiv region. ECF is a dry mixture of homogeneous consistency in powder and fine grains with taste, smell, and color inherent to raw materials, made by grinding grain parts (endosperm, aleurone layer, fruiting membranes, with pre-removed embryo) and hot extrusion method. Wheat flour of the highest grade with a crude gluten content of 23.0 ±0.4% and a crude gluten deformation value of 60 ±1.1% etc., potato starch, chicken eggs, white crystalline sugar. The characteristics of the batches of flour used in the experimental studies are shown in Table 1.

Research methods

Standard, conventional, special, and modified physics and chemical, microbiological, and organoleptic research methods were used to perform the work.

Wheat and FCE quality were established in each consignment based on the results of the average sample analysis according to **DSTU 3355-96, DSTU ISO 9001:2015 IDT**.

Sampling and sample preparation for laboratory testing of The raw materials was carried out in accordance with a single methodology for the study of domestic food. The test and control samples were prepared from one batch of raw materials (Table 2).

The experimental part of the work was carried out in the research laboratories of the Department of Food Technology, TNTU University, and the laboratory of grain quality at the Institute of Plant Growing named after V.Ya. Yurjev of the National Academy of Agrarian Sciences of Ukraine.

The prototyping technology includes the following operations: the egg-sugar mass is beaten to a 2.5 – to 3-fold increase in volume, ie approximately 30 – 40 × 60 s. Then gradually add the premium wheat flour mixed with starch or extruded corn flour. The mixing was carried out in the developed machine (Figure 2) with a duration not exceeding 35 s.

Ready-made biscuit dough is baked immediately in capsules and on sheets, as the biscuit dough settles when stored. Biscuit dough was placed in shapes at ¾ of their height because when baking it increases in volume Figure 3.

Baking biscuit dough was carried out at a temperature of 200 – 210 °C. Baking time in capsules was 50 – 60 × 60 s. Baked biscuit cake was cooled 20 – 30 × 60 s and removed from the molds. The biscuit semi-finished product is left at 8 – 10 × 60 c2 for standing, after which it is possible to cut and carry out the following technological operations.

The structural and mechanical qualities of the dough include elastic and flexible and viscous and plastic qualities. The elastic and flexible qualities of the dough were determined using a farinograph "Brabender", which describes the process of dough formation and its behavior under continuous machining conditions, and the viscous and plastic qualities on the alveograph "Chopin". The foam resistance of the egg and sugar mixture with the addition of the test flour mixtures was determined as the ratio of the foam column height after holding for 24 × 602 s at a temperature of 18 – 20 °C to the total foam column height of the sample, expressed as a percentage, calculated by the formula: (1)

$$СП = \frac{Bn^{24\cdot 60^2}}{Bn} \times 100\% \quad (1)$$

Where:
СП is foam resistance,%;
Bn60 is the height of foam column 24 × 602 s after cessation of whipping, m;
Bn is the initial foam height, m.

The specific volume of products was calculated as the ratio of their volume to mass ($m^3.kg^{-1}$). Baking was defined as the difference between the weight of the dough before baking and the weight of the finished semi-finished product.

The degree of batch penetration of baked semi-finished products after baking and during storage was examined on a Labor Penetrometer (Hungary) according to the standard method. Microscopic studies of the structure of the product were performed **(Rochow and Rochow, 2012)** using a digital binocular microscope series "MicroMed" equipped with a built-in lighting system. The micrographs of the samples were taken at the following magnifications: 40 times, 100 times, 400 times, 1000 times. The porosity of the samples of the biscuit cake **(Stadnyk et al., 2015)** – control (basic biscuit) and extruded corn flour were carried out by measuring the effective pore diameters on the biscuit cross sections by visualizing the microstructure of the biscuit, processing the digital images.

Statistical analysis

The assessment of the basic regularities of changing the qualities of biscuit dough is based on the optimal parameters and modes of its formation. They are based on the initial values of the process, which determines the probability of the obtained results of the structure required to describe the consumer properties. The features of the influence on the treated environment in the formation of the foamy structure of the dough and the conditions to achieve rational parameters of the mixing in the working compartment, it is possible to predict the performance of the mixer. To clarify the role of individual factors of the system, planning and setting up of computational experiments were performed to obtain the corresponding regression equations. Determination of the influence of the design parameters of the mixer (independent factors xi) on the foaming process (Q), conducted full factorial experiments FFE 3^3. They aim to determine the magnitude of productivity and to establish its effect on the structure when mixing components that depend on changes in the three main factors: the frontal surface of the plate working body α, the distance between the plates (step) t and the speed of rotation of the working body *n*. The construction of the plan-matrix of the planning of experiments indicated the conditional factors of the upper, lower, and zero levels of variation, respectively +1, -1, 0. The results of the encoded factors and their levels of variation are shown in Table 3. The construction of this table is as follows. The input values of the FFE 3^3 variable factors are:

-the speed of the plate n (rpm), which is encoded by the index x_1;

-the angle of attack of the frontal surface of the stroke of the plate α (deg) encoded by the index x_2; is the step between the plates t (m) encoded by the index x_3.

Statistical analysis of the results was performed to analyze the dependencies obtained with the help of the software package on the PC "Statistica 6.0". Determinations of the mathematical variance of random variables D were estimated by standard methods. The regression dependence of the performance on the change in the speed of the shaft n, the angle of attack of the frontal surface of the stroke of the plate α, deg and the pitch of the plates t, ie Q X_1 X_2 X_3 = f (n α t), for the encoded values of the factors for mixing has the form:

$$Q_{(n,\propto,T)} = 2.185 - 0.0031x_1 - 0.114x_2 + \\ +1.559x_3 + 0.000012x_1x_2 + \\ +0.000031x_1x_{3+} + 0.00036x_2x_3 \\ +0.0000023x_1^2 + 0.00071x_2^2 - 0.0723x_3^2$$

For the natural values of the factors of the regression equation (4.1) for mixing takes the form:

$$Q_{(n,\propto,T)}=1.224+0.0061n-0.106\propto_1+1.591T_2+0.00071\propto_1^2-0.0723T_1^2$$

The obtained regression equation can be used to determine the performance of the mixer for different formulations (Q), depending on the change of speed of the shaft with the working bodies n, the angle of attack of the frontal surface of the stroke of the plate α and the step between the tariaks t within the following limits of change of input factors:

$348 \leq n \leq 696$ (rpm); $30 \leq 1\ \alpha \leq 60$ (deg); $0.9 \leq t \leq 0.14$ (m).

From the obtained regression equations it was found that the factors that influence x1, x2, (n, α), and their combination are the biggest influence factors on the

change in performance. Productivity is reduced to 20% by increasing the value of factor x2 (α) (Table 3).

By increasing the speed of the shaft and decreasing the angle of attack of the frontal surface of the plate stroke, the performance of the mixer is increased.

The response surfaces and their two-dimensional sections are built on the regression equations obtained to determine the performance from the change in the two factors for x3 = const. The results of the performance dependencies obtained with the Statistica-6.0 program are shown in Figure 4 and Figure 5.

The statistical processing of the experimental results and the obtained regression dependencies adequately reproduce the research processes for determining the mixing performance of the components which is the quality of the whipping of the foaming biscuit dough.

Figure 4 and Figure 5 show that as the shaft speed increases, the value of productivity increases, with the highest productivity being achieved when mixing gluten-free flour. Minimum performance value with minimum shaft speed and maximum attack angle of the frontal surface of the stroke of the plate.

RESULTS AND DISCUSSION

To analyze consumer properties and establish rheological dependencies, the studies were directed in such a sequence as to justify the feasibility of using an ECF.

Features of the methods of mixing we have chosen, the composition and state of the liquid phase of the dough, allow us to solve several technological problems. First, the liquid phase is the binding component for the formation of a continuous dough network. Second, the properties of the liquid phase actively influence the process of foam formation and its retention in the dough. Also **(Lisovska, Chorna and Dyakov, 2016)**, it is an important flavor component of the cupcake recipe.

The authors' research **(Kobets et al., 2015)** established the effect of a mixture of fibers and emulsifiers on the gluten complex of wheat flour by physicochemical quality indicators of the biscuit semi-finished product. To regulate the set quality of confectionery dough, the authors **(Mancebo, Rodriguez and Gómez, 2016)** believe that you can use different ratios of starch and protein.

The results of the authors' research **(Egorova and Reznichenko, 2018; Dorohovych, Hrytsevich and Isakova, 2018)** reflect the influence of technological and design factors on the model systems of biscuit semi-finished products. They analyzed the effect on the volume and shape of the finished products, the structure of their porosity, as well as the nature of the structural, and mechanical properties of the dough, the actual time of dough formation, its stability, degree of depression, consistency and elasticity.

Thus, research **(Haliasnyi, Gavrish and Shanina, 2018)** is aimed at finding structure-forming components for gluten-free flour confectionery. They conducted a comparative analysis of changes in the fractional composition of flour dough proteins with different types of flour raw materials and liquid phases. Problems related to changes in the structural and mechanical properties of the environment and the impact of structural elements and methods of the process, discussed in detail **(Szwedziak et al., 2019)**, taking into account empirical data, theoretical dependences, and the results of physical experiments.

The specificity of the production of gluten-free dough cakes by intensive mixing (whipping) is that the resulting foam structure is subjected to unwanted external influences that lead to a decrease in its stability. Such factors include mixing the whipped mixture with flour and placing the dough in molds. In such circumstances, it is important not only to obtain a foam system with the required characteristics but also to preserve them during the technological process. Accordingly, in our view, it is important to comprehensively investigate the foaming ability and foam resistance to structure destruction. It should be noted that obtaining a well-loosened dough structure is the first but not the only one technological task.

The issue **(Lisovska, Chorna and Dyakov, 2016)** of the preservation of the formed structure, which determines the corresponding rheological properties of the dough is also important. It should be noted **(Lisovska, Chorna and Dyakov, 2016; Dickinson, 2015)** that, unlike the traditional confectionery foam (for example, in the preparation of biscuits, where the amount of sugar is much higher), in gluten-free cupcakes, recipe sugar level is much lower. Therefore, the issue of effective foaming is extremely important.

In their studies **(Murtini and Putri, 2017)** to improve the volume as emulsifiers egg yolk substitute was added that is a suspension of edamam (Glycine max L. Merill). Five different proportions of edamam suspension were added to the dough and their effect on specific volume, porosity, water content, oil absorption, hardness texture, and elasticity was observed. It should be noted that many works **(Lisovska et al., 2020)**, considered the foam structure of the dough for cupcakes with the establishment of the main technological and design parameters of the interaction of prescription mixtures. They substantiated the quality of the cake with the ratio of the main recipe components: egg content (51%); sugar content (24.4%) and content of extruded corn flour (24.6%).

Along with the prescription components, the duration of the whipping and the speed of rotation of the working organs (machine productivity) are influenced by the foam volume and its dispersion, the foaming ability, and the foam stability.

As the whipping time increases, the volume of foam also increases; the whipping rate is directly proportional to the foam dispersion. We chose the traditional mode at high foam whipping.

Table 1 Chemical composition of extruded corn flour and wheat flour of the highest grade, %.

Product	Moisture content	Protein content	Fat content	Starch content	Ash content	Fiber content
Extruded corn flour	9.0 ±0.01	6.1 ±0.02	8.1 ±0.02	70.9 ±0.03	4.8 ±0.03	1 ±0.02
Wheat flour of the highest grade	14.5 ±0.03	11.4 ±0.05	1.08 ±0.04	67.7 ±0.05	0.5 ±0.03	0.1 ±0.01

Table 2 Formulation of experimental samples of biscuit cake.

Raw materials	solids content (SC), %	Cost of raw materials per 100 kg semi-finished product, kg			
		Biscuit semi-finished product Control		Gluten-free cookie cake	
		In kind	In DS	In kind	In DS
Wheat flour of the highest grade	85.50	28.12	24.04	-	-
Extruded corn flour	91.0	8.07	7.34	30.58	27.82
Potato starch	80.00	6,94	5.55		
Eggs	27.0	57.85	15.62	63.44	17.12
White sugar	99.7	34.71	34.65	30.26	30.16
Altogether:		135.69	87.2	124.28	75.1
Output		100.0	87.0	100.0	75.00

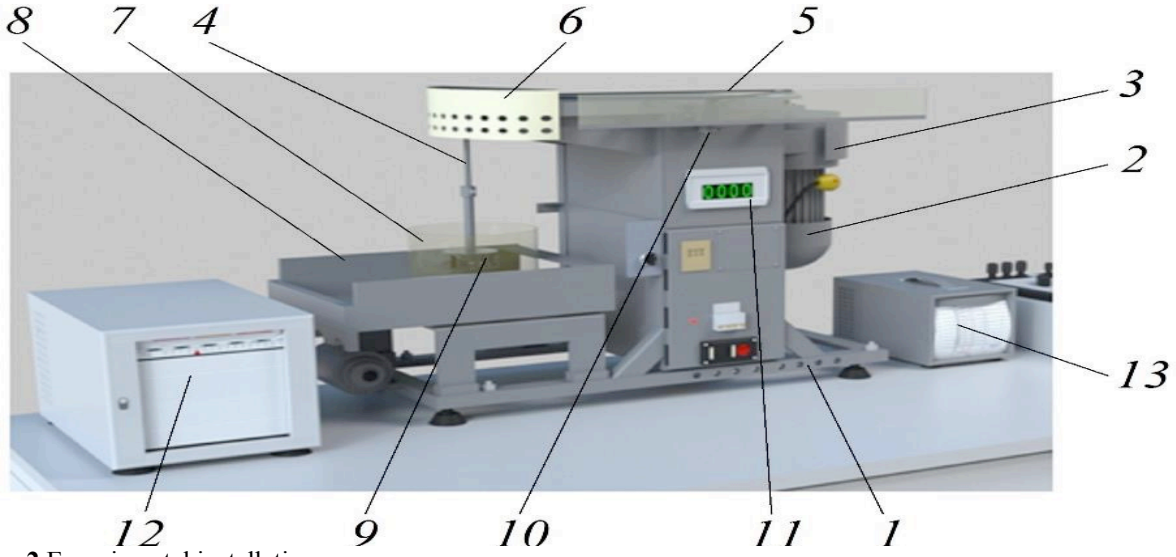

Figure 2 Experimental installation.
Note: 1 – frame; 2 – electric motor; 3 – coupling; 4 – shaft; 5 – V-belt transmission; 6 – the casing; 7 – a bowl; 8 - panel; 9 – disc working bodies; 10 – speed controller; 11 – electronic tachometer; 12 – potentiometer; 13 – wattmeter.

Figure 3 Biscuit dough placed on forms and on a pastry sheet.

Table 3 Characterization of factors and the significance of their levels for experimental performance studies.

Factor designation	code	Name of factor	Factor levels values
x_1		Auger speed n, rmp	348-552-696
x_2		tilt angle of unloading line a_1 degree	30-45-60
x_3		Auger step T_2, m	0.09-0.115-0.14

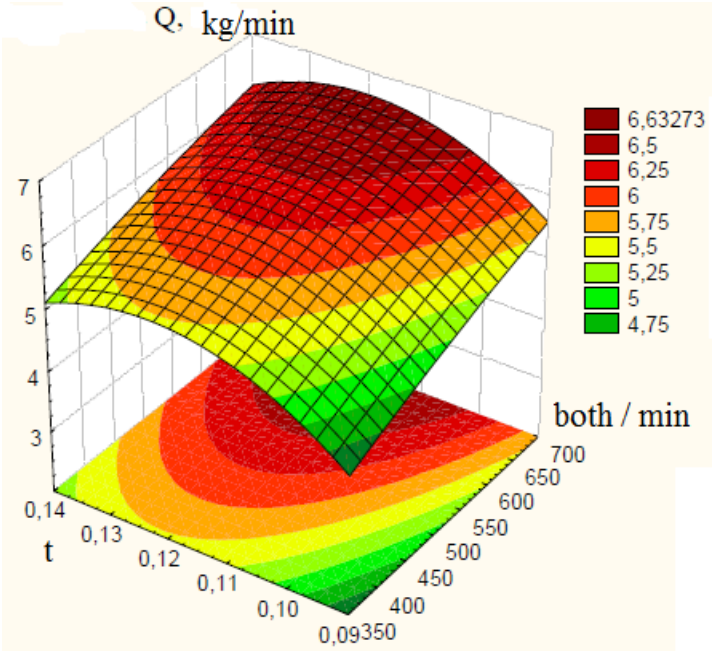

Figure 4 Response surface of the dependence of the mixer Q performance on the shaft speed and the pitch between the plates (α1 = 45 deg).

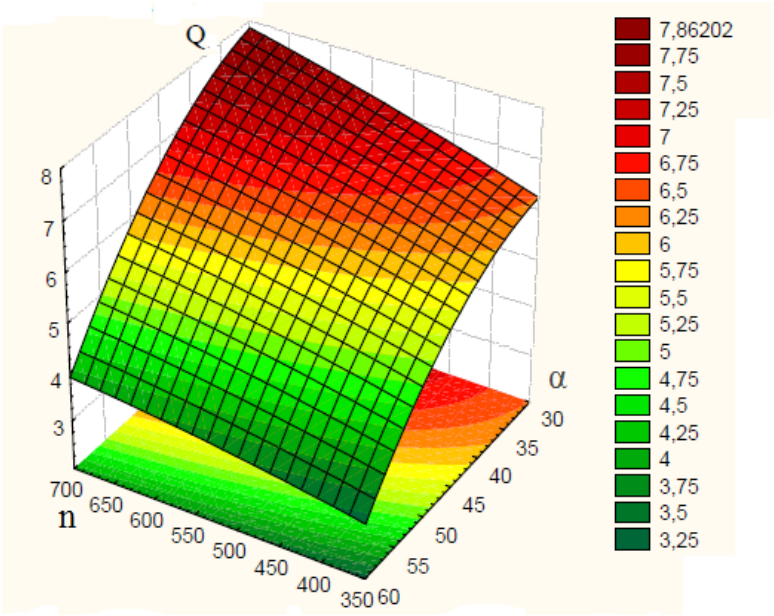

Figure 5 Response surface of the dependence of the performance of the mixer Q on the speed of the shaft and the angle of attack of the front surface of the plate stroke (t = 0.115 m).

Because the offered introduction of ECF to the recipe mixture affects the stability of the system, we conducted a set of experiments to study the dependence of the effective viscosity of biscuit dough samples with the addition of ECF on the velocity and shear voltage. In the tested mixtures, the moisture-holding capacity increases for the sample containing 20% by weight of corn extruded flour by two and a half times, and three times for the sample with extruded flour by 100% by weight. This tendency of change is explained by the swelling of whole starch grains due to the absorption and retention of moisture. With an increasing proportion of corn extruded flour in the flour mixture, the dough density increases and the optimum value is in the range of 0.444 – 0.446 $kg.m^{-3}$. An increase in the flour of corn extruded above 20% by weight leads to a considerable density of the dough, but in the absence of wheat flour of the highest grade indicates the possibility of creating a biscuit gluten-free semi-finished product.

From Figure 6 it is confirmed that the foam stability depends on the flour used. The use of corn extruded flour significantly affects the stability of the sponge dough foam. Foam resistance monotonically increases almost 1.5 times with the complete replacement of wheat flour, which corresponds to a large volume and a thin uniform porosity of the biscuit cake. This ability will help to stabilize the foam of the biscuit dough during mechanical influences during its molding.

The stability of the dispersed system is due to the structural and mechanical properties of the adsorption layers and the thermodynamic stability of the liquid layers. These adsorption layers slow the flow of liquid in the pellicles, reducing the rate of their thinning. This is confirmed in the work **(Zhou et al., 2015)** that revealed the effect on the moisture-holding capacity of the system of flour mixtures. At the same time, it was noted **(Zhou et al., 2015)** that free starch polymers dissolve to form a dispersed system.

Also, these layers give the foam pellicle a high structural viscosity and mechanical strength, creating a framework that gives the foam certain physical and chemical properties of the solid.

An important rheological characteristic of biscuit dough as the foam is the viscosity that acts as a structural and mechanical barrier in the formation and destruction of the foam structure. The dependence of the maximum viscosity of the suspension on the contents of the ECB in flour mixtures is shown in Figure 7.

The stability of the dispersion system, which is a biscuit dough largely due to the strength and resistance to loading, i.e. the creation of an elastic frame, which gives the system certain physical and chemical properties of the solid. Analysis of the studies showed that when adding 5 and 10% of the ECF there is a maximum viscosity, which indicates the starch binding during a large amount of water. When using 100% ECF (Figure 7), the viscosity decreases, because the starch binds a small amount of water during swelling and pasteurization. The water is free in the dough and released when coagulated with protein substances, which promotes the formation of a wetter crumb cake mix.

In the works of authors **(Lisovska, Chorna and Dyakov, 2016; Murray and Ettelaie, 2004)**, it is noted that the schematic structure of the foam is the packing of gas bubbles with thin pellicles of the main fine particulate filler, which is covered with pellicle substance with surfactants. Figure 9 shows the comparative characteristics of the microstructure of the following samples: wheat flour (WF) and extruded corn flour (ECF) in the ratios: a) WF – 100 wt.%; b) WF: ECF – 80:20 wt.%; c) ECF – 100 wt.%.

The thermodynamic correlation relative to the gas phase makes it possible to determine its volumetric deformation, which is the basis for determining the rigidity of the system.

Many studies **(Sandri et al., 2017)** proved that the moisture content of the dough in the case of rice and corn flour should be reduced compared to the moisture content of the dough with wheat flour, in the case of buckwheat flour it should be increased to obtain products high quality. They established and calculated the range of change in viscosity with increasing shear stress according to the viscosity curves.

For example, in the study **(Murray and Ettelaie, 2004)**, it is noted that the destruction of the air bubble film is directed toward the larger bubble because its pressure is lower than that of the small bubble.

On these microstructures of biscuit dough (Figure 9) of prototypes, it has the appearance of foam with an existing and uniform distribution of air bubbles, which later form a porous structure of biscuit semi-finished products. The sizes of the formed air bubbles with the content of wheat flour and starch have a large difference in diameters, that is, some bubbles are almost twice the size of the others. In the sample of biscuit dough using ECF 100 wt.% Figure 9 (4) foam bubbles are practically the same size and channels are formed between them, which help to equalize the air pressure in the middle of the foam biscuit dough system. The sample with an ECF content of 20 wt.% is also characterized by the same size of gas bubbles, which contributes to the stabilization of the foam system.

This leads to an improvement in the structural and mechanical characteristics of ready-made biscuit products.

After baking the samples (Figure 8), a semi-finished biscuit with a volume at the level of the sample of wheat flour with a fine uniform porosity was obtained. The use of 20 wt.% (Figure 8, number 2) and 100 wt.% (Figure 8, number 3) corn extruded flour contributes to the formation of a fine porous structure of biscuit dough.

Thus, the total area of large pores with a size of 0.7 – 1.5 mm in the sample with WF – 100 wt.%. It is equal to 15.36 mm^2 in the field of view and in the test samples variant WF: ECF – 80:20 wt.% and ECF – 100 wt.% is 3.69 and 2.94 mm^2 accordingly.

At the same time, the number of small and very small pores (less than 0.5 mm) is significantly increasing, mostly in the sample with ECF – 100 wt. %. The obtained data are correlated with the data on foaming capacity, which is the highest ECF that is 100 wt.%.

The above indicates that the proposed additives to improve gluten-free dough, improve the porosity of the foamy structure, forming a fine porous structure of the cake. This effect is due to the ability of high-quality deformation impact of the working bodies of the machine.

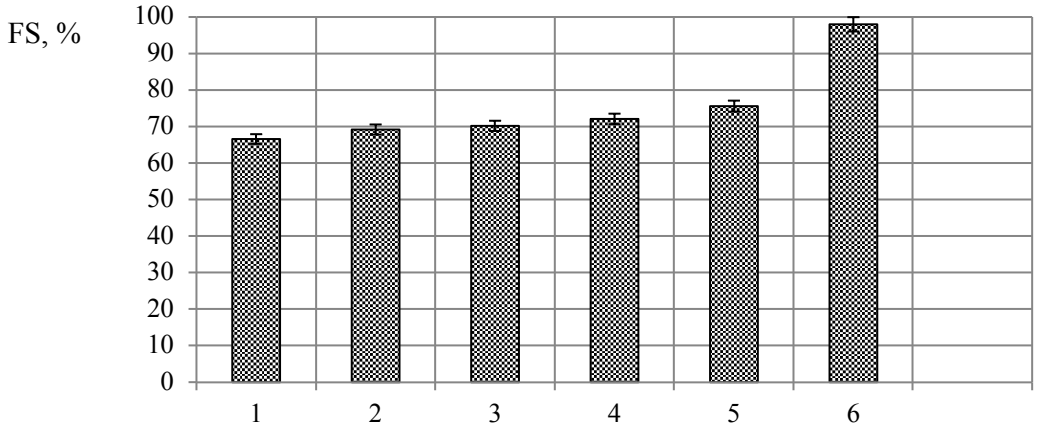

Figure 6 Dependence of biscuit dough foam stability on ECF content.
Note: 1 – WF:ECF– 100: 0 (control); 2 – WF: ECF – 95: 5 wt.%; 3 – WF: ECF – 90: 10 wt.%; 4 – WF: ECF – 85:15 wt.%; 5 – WF: ECF – 80:20 wt%; 6 – WF: ECF – 0: 100 wt.%.

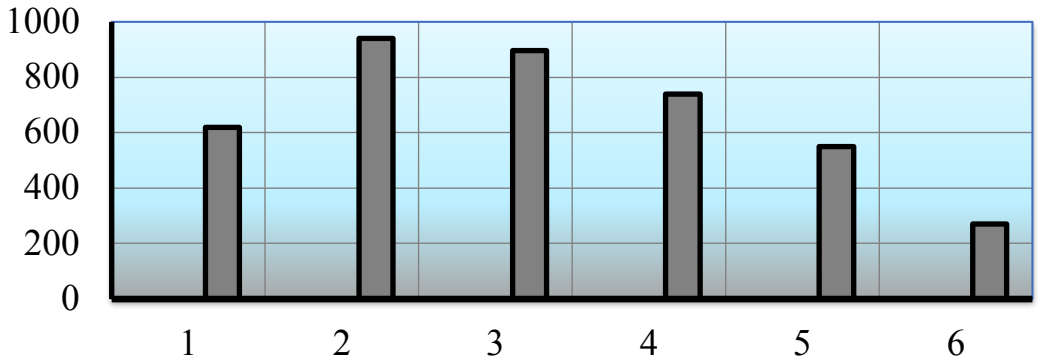

Figure 7 Dependence of the maximum system viscosity on the content of the ECB in flour mixtures.
Note: Wheat flour of the highest grade and ECF in the ratios: 1 – WF: ECF – 100: 0; 2 – WF: ECF – 95: 5; 3 – WF: ECF – 90:10; 4 – WF: ECF – 85:15; 5 – WF: ECF – 80:20; 6 – WF: ECF – 0: 100.

Figure 8 Photos of the surface and porosity of the biscuit cakeusing.
Note: ECF: 1) WF – 100 wt.%; 2) WF: ECF – 80:20 wt.%; 3) ECF – 100 wt.%.

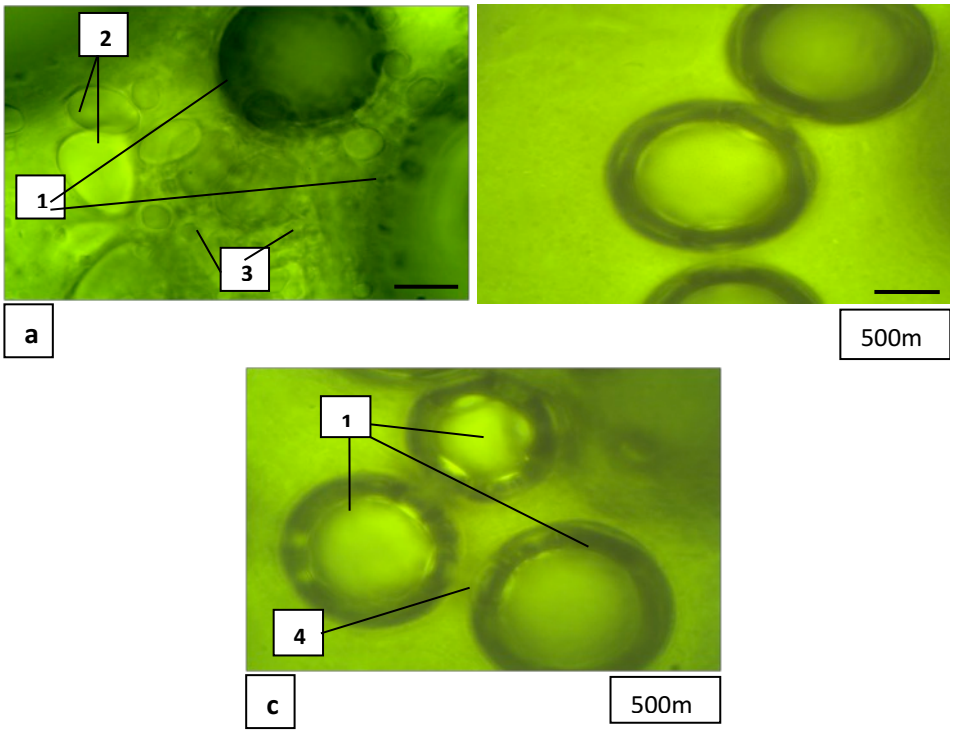

Figure 9 Microstructure (1: 1000) of biscuit dough samples.
Note: Dough samples containing: WF and ECF in the ratios: a) WF – 100 wt.%; b) WF: ECF – 80:20 wt.%; c) ECF – 100 wt.%; 1 – air bubbles; 2 – potato starch grains; 3 – wheat flour starch grains; 4 – channels between air bubbles.

CONCLUSION

Therefore, it can be concluded that the use of the obtained results allows regulating the technological properties of flour mixtures, depending on the concentration of them in the ECB and recommend them in the production of confectionery flour health products.

Stabilization of the rheological properties of the foam system of biscuit gluten-free dough is achieved due to the properties of partially pasteurized starch of extruded flour. Gluten-free biscuit recipe composition is optimized: 51% of egg product content; 24.4% of sugar content and 24.6% of flour content of extruded corn.

The presence of stable hydrodynamic parameters of gas-liquid systems under the action of the geometry of the machine gives reason to consider them metastable. The motive factor is determined by the product of the external pressure on the cross-sectional area of the working room, and the opposing force in the deformation of the gas-liquid medium is the elastic forces and the forces of internal friction.

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RELATIONSHIP BETWEEN THE ACTIVITY OF GUAIACOL PEROXIDASE AND THE CONTENT OF PHOTOSYNTHETIC PIGMENTS IN TEA LEAVES

Nataliia Platonova, Oksana Belous

ABSTRACT
The dynamics of guaiacol peroxidase and photosynthetic pigments in 3-leaf sprouts (flushes) of tea plants were studied. The presence of declines and peaks in the activity of the enzyme associated with the meteorological conditions of each month was noted. It is shown that there is a direct relationship between the increase in enzyme activity and hydrothermal factors. The most significant correlation was found between the activity of GPO in a 3-leaf tea flush and the amount of precipitation (r = 0.86). The highest activity of guaiacol peroxidase during the entire vegetation period is distinguished by the Sochi variety and form 582. The lowest activity was observed in forms 3823 and 2264, which indicates a low intensity of redox reactions in these plants in stressful situations. Determining the dynamics of the pigment complex revealed its dependence on hydrothermal factors. Studies have shown that precipitation is a significant factor affecting the pigment complex of tea plants. It was found that the largest amount of green pigments is synthesized by leaves at the beginning of active vegetation (May). The participation of the pigment apparatus in the adaptation of the tea plant is directly related to carotenoids, the increase in the number of carotenoids coincides with the period of drought. In the content of photosynthetic pigments and the activity of guaiacol peroxidase manifest genotypic features. The revealed patterns are common to all tea plants.

Keywords: tea; chlorophyll; carotenoid; guaiacol peroxidase; air temperature; precipitation; correlation; stability

INTRODUCTION
One of the most important problems of phytophysiology is the study of the mechanisms of plant resistance to adverse environmental factors **(Foyer and Noctor, 2000; Kareska, 2009; Belous and Platonova, 2018a)**. One of the consequences of stress is the formation of reactive oxygen species (ROS). In a comfortable state of the cell, the ROS content is maintained at a low level, thanks to the work of special antioxidant enzyme systems that utilize reactive oxygen species. The amount of ROS that is not eliminated by enzymes, accumulated in the cell, and causing damage to it, is called oxidative stress. Oxidative stress is a non-specific reaction of plants to the action of stress factors **(Kareska, 2009; Belous and Platonova, 2018c)**. Under the action of stressors, the content of ROS in cells increases significantly, especially in heat-loving plants, which include all subtropical crops, such as tea.

Several authors noted an increase in the activity of peroxidases, especially when inactivating certain antioxidant enzymes, such as catalase **(Krasensky and Jonak, 2012; Eremchenko, Kusakina and Luzina, 2014; Kaur and Asthir, 2017; Belous, Klemeshova and Malyarovskaya, 2018; Belous and Platonova, 2019)**. It is no accident that recently special interest has been directed to the study of peroxidases – enzymes of the class of oxidoreductases that catalyze the oxidation of various substances with the help of hydrogen peroxide. The level of peroxidase activity in plant organs is used to characterize their functional state in response to extreme environmental factors **(Bukhov et al., 2001; Kapchina-Toteva et al., 2004; Ladygin and Shirshikova, 2006)**. An increase in peroxidase activity in the photosynthetic organs of plants indicates active photosynthesis during stress.

An important question for us is related to the fact that the peroxidase substrates are phenolic compounds, ascorbic acid, a number of aromatic acids, carotenes, etc. **(Kasote et al., 2015)**. All these compounds are significant components of the antioxidant system of tea and determine its nutritional value **(Fedotova, 2009; Mulgund, Doshi and Agarwal, 2015; Belous and Platonova, 2018b)**. But the main substrate is phenols, which under the action of the enzyme are oxidized to polyphenols and quinones, which are strong oxidizers **(Rogozhin, 2004)**. Quinones are capable of polymerization, resulting in dark-colored compounds. This process is especially active at the stage of enzymatic oxidation of raw materials (3-leaf sprout) in the production of black tea.

The effectiveness of the photosynthetic apparatus in stressful conditions is due to the peculiarities of the

pigment complex. This is one of the most important indicators of the adaptive potential of plants since carotenoids are important components of the antioxidant system and play an important role in protecting plants from photo-oxidative processes. Carotenoids take part in redox processes; normalize oxygen consumption by plant tissues. These pigments are effective antioxidants because they absorb singlet molecular oxygenized radicals.

The composition of the antioxidant system of tea includes the main components that determine the taste of the finished product, the importance of these substances for humans is undeniable and the study of the patterns of their accumulation is necessary **(Belous and Platonova, 2018b)**. Tea plants in the conditions of subtropical Russia are often exposed to stress factors during the active vegetation period; the study of the regularities of the formation of components of the antioxidant system of tea is undoubtedly relevant.

The picture of tea sprout is presented in Figure 1, black tea from Krasnodar sprout is presented in Figure 2 and the tea harvesting on the experimental plantation is presented in Figure 3.

Scientific hypothesis

There is a relationship between the activity of guaiacol peroxidase and hydrothermal factors.

A decrease in the activity of guaiacol peroxidase is unfavorable for the stability and quality of tea plants.

Hydrothermal factors influence the content of photosynthetic pigments.

The ratio of the amount of chlorophyll to carotenoids indicates the degree of adaptation of tea plants to adverse conditions.

MATERIAL AND METHODOLOGY

Objects of research – 3-leaf sprouts (flushes) of tea plants (*Camellia sinensis* (L.) O. Kuntze) varieties of Sochi and forms of Institute selection 3823, 582, 855, and 2264, grown on the experimental collection and breeding site of the Russian Institute of Floriculture and Subtropical Crops in the village Uch-Dere (Lazarevsky district, Sochi, Russia). Control – Colchida variety. The plantations are located at an altitude of 280 m above the sea. Soils in the Krasnodar region where tea plantations are located are brown forest slightly unsaturated. In the humid subtropical zone of Russia, the driest period coincides with the period of active vegetation – June and August. Weather conditions in recent years differ significantly from the long-term norm, both in terms of precipitation and air temperature. Thus, the precipitation deficit in May – August is on average 28.5 – 87.0 mm; the absolute maximum temperature is in the range of 35.5 (in June) – 32.3 (in August) °C, at that time, an average monthly average temperature of 22.3 ±1.2 °C. The average duration of sunshine is 244 hours per month (May – August), which corresponds to almost completely cloudless days in summer.

The selection of flushes was carried out in 3-fold field repetition in the period from May to August 2017 – 2019. The determination of laboratory analyses was performed in 3-fold repetition in the laboratory of plant physiology and biochemistry. All chemicals were analytical grade and obtained from LENREACTIV (Russia).

Determination of pigments

We used a spectrophotometric method for determining the content of chlorophyll and carotenoids with the extraction of pigments with 96% ethanol and using the calculated formulas of Smith and Benitez **(Shlyk, 1971)**. The optical density of extracted pigments was measured using a PE-5400VI spectrophotometer, manufactured by EKROSHIM LLC (Russia) at a wavelength of 665 and 649 nm for chlorophylls a and b, and 440.5 nm for carotenoids in cuvettes with a layer thickness of 10 mm.

Determination of the activity of guaiacol peroxidase

The activity of guaiacol peroxidase (GPO) was determined by the spectrophotometric method, taking into account the rate of utilization of hydrogen peroxide in the reaction mixture, into which the plant material is introduced. The intensity of utilization of hydrogen peroxide was judged by the rate of extinction reduction at a wavelength of 440 nm against the phosphate buffer (pH 6.7) **(Vorobyov et al., 2013)**.

Statistical analysis

The arithmetic mean values of the measured values and their standard deviations are shown in Figures and Tables. The correlation between the samples was estimated by calculating the Spearman rank correlation. To check the significance of correlation and estimate statistical values, an analysis was performed using the ANOVA package in STATGRAPHICS Centurion XV (version 15.1.02, StatPoint Technologies) and MS Excel 2007. Statistical analysis included a one-dimensional analysis of variance (a method for comparing averages using variance analysis, t-test). The significance of the difference between the average values at $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

When determining the activity of guaiacol peroxidase in freshly harvested 3-leaf tea flushes during the active vegetation period, the presence of declines and peaks in the activity of the enzyme associated with the meteorological conditions of each month was noted (Figure 4 and Figure 5). At the beginning of the growing season (May), the activity of the enzyme was low – in the range of 0.363 to 0.607 unit.g^{-1}·sec.

In June, there is a decrease in activity, which, however, is not significant and is due to the biological characteristics of the tea culture. After the May surge in growth processes in tea plants, there is a period of rest, in which the metabolic processes are somewhat slowed down. As a rule, in the future, in our zone there is a stressful period under hydrothermal conditions, which affects the functional state of plants; in particular, we can note an acute water deficit. This leads to the manifestation of oxidative stress, which is expressed in an increase in the activity of GPO, as a non-specific reaction to a stress factor **(Birben et al., 2012; Keshari et al., 2015; Ognik et al., 2016)**.

Figure 1 Tea sprout.

Figure 2 Black tea from Krasnodar sprout.

We performed a correlation analysis that showed a direct relationship between increased enzyme activity and hydrothermal factors (Table 2). At the same time, the most significant correlation was found between the activity of GPO in freshly harvested 3-leaf tea flushes and the amount of precipitation (r = 0.86).

However, the dynamics of enzyme activity in a 3-leaf sprout is variety-specific (see Table 1). The highest activity of guaiacol peroxidase during the entire vegetation period is characterized by the Sochi variety and form 582 (about 0.56 unit.g^{-1}·sec).

The lowest activity was observed in forms 3823 and 2264, which indicates a low intensity of redox reactions in stressful situations, under the influence of changing environmental factors on plants. Tea sprouts are used to prepare a drink whose nutritional value is made up of substances formed both in the process of photosynthetic reactions and in the process of processing raw materials, the basis of which is redox enzyme reactions **(Chen and Asada, 1989; Pandey et al., 2017; Skhalyakhov et al., 2019)**. Given this fact, a lower level of activity of guaiacol peroxidase is a negative phenomenon, both for the stability of the plant itself and for the quality of the finished tea.

One of the indicators of plant response to changes in hydrothermal conditions is the quantitative content of chlorophyll and carotenoids – the main photoreceptors of the photosynthetic cell **(Fedotova, 2009; Eremchenko, Kusakina and Luzina, 2014)**. Stress factors, including lack of precipitation and high positive temperatures, significantly increase the probability of photo-oxidative damage in chloroplasts **(Foyer and Noctor, 2000; Mittler, 2002; Kapchina-Toteva et al., 2004; Ladygin and Shirshikova, 2006; Kareska, 2009; Nikolaeva et al., 2010)**. Only by studying the pigment system of plants can we fully identify the biological and adaptive capabilities of the culture. As shown by research, a significant factor that affects the pigment complex of tea plants is the amount of precipitation.

Determination of the dynamics of the pigment complex showed its dependence on hydrothermal factors: the largest amount of green pigments is synthesized by leaves at the beginning of active vegetation (May). In July, after short rains of a stormy nature that do not cover the water deficit in the soil, growth processes in tea resume, but less actively than in May. There is an increase in new sprout; green pigments which are intensively synthesized in leaf blades, which manifest itself in an increase in their number. In August, the drought increases, which affects not only the inhibition of growth processes but also a significantly lower accumulation of the green group of pigments (LSD ($p \leq 0.05$) = 0.12).

Figure 3 Tea harvesting on an experimental plantation (Sochi, Russia).

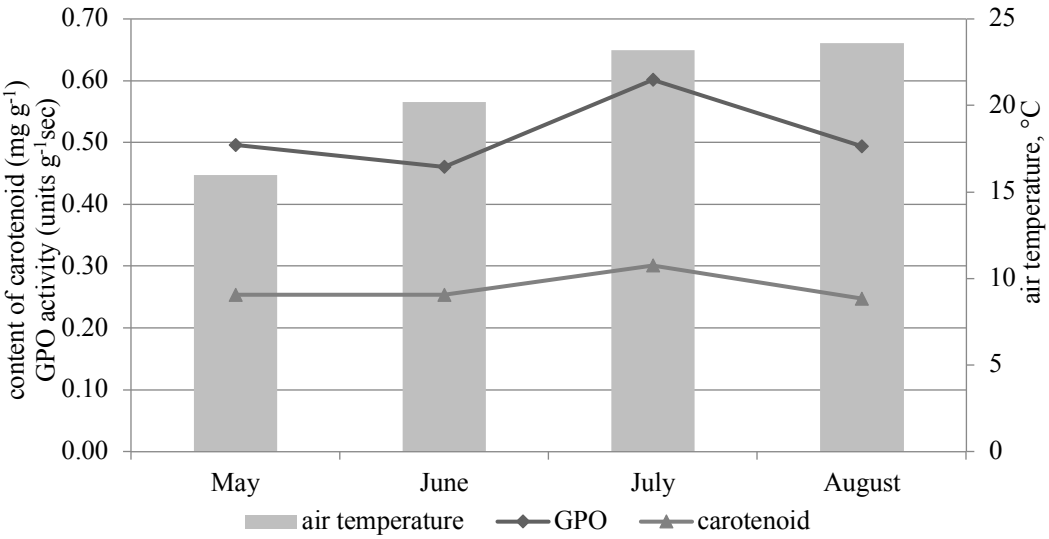

Figure 4 Dynamics of AOS components in a 3-leaf sprout *Camellia sinensis* depending on temperature conditions of vegetation.

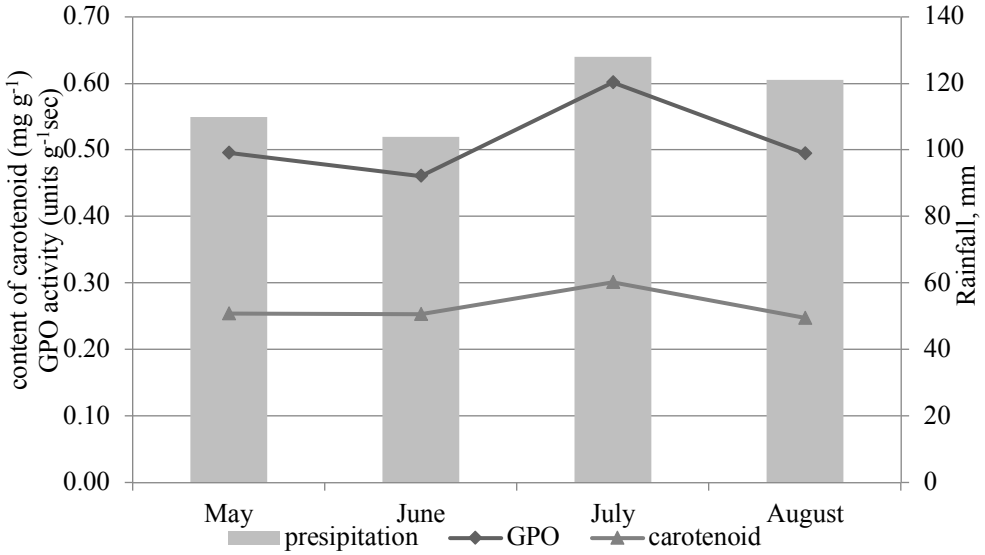

Figure 5 Dynamics of AOS components in a 3-leaf sprout *Camellia sinensis* depending on the amount of precipitation during the growing season.

Table 1 Varietal features of accumulation of components of AOS by 3-leaf *Camellia sinensis* sprout.

Variety/form	The activity of GPO, unit.g^{-1}·sec	V,%	Sum of chlorophylls, mg. g^{-1}	V,%	Sum of carotenoids, mg. g^{-1}	V,%
cv. 'Colchida'	0.52 ±0.10	8	1.09 ±0.07	7	0.29 ±0.02	9
cv. 'Sochi'	0.56 ±0.11	17	1.04 ±0.07	7	0.29 ±0.02	6
f. 3823	0.56 ±0.17	20	0.86 ±0.09	11	0,24 ±0.03	14
f. 582	0.45 ±0.05	39	0.97 ±0.10	10	0.26 ±0.03	11
f. 855	0.54 ±0.05	10	0.92 ±0.09	9	0.25 ±0.03	13
f. 2264	0.46 ±0.01	11	0.95 ±0.11	11	0.26 ±0.03	13
HCP ($p \leq 0.05$)	0.05		0.05		0.01	

Table 2 Coefficient of pair correlation between the studied parameters and hydrothermal factors.

Parameters	GPO, unit.g^{-1}·sec	Sum of chlorophylls, mg. g^{-1}	Sum of carotenoids, mg. g^{-1}
GPO, unit.g^{-1}·sec	1.00	-	-
Sum of chlorophylls, mg. g^{-1}	-0.26	1.00	-
Sum of carotenoids, mg. g^{-1}	0.94	-0.48	1.00
Air temperature, °C	0.42	0.54	0.38
Amount of precipitation, mm	0.86	0.27	0.68

The participation of the pigment apparatus in the adaptation of the tea plant is directly related to carotenoids (Figure 5); as can be seen from Figure 5, a slight increase in the number of carotenoids coincides with the period of drought and the natural June decay of growth processes. In August, with significant water scarcity there has been a sharp increase in the synthesis of carotenoids, because of the continuous drought period, followed by increasing the temperature to 30 degrees or more, and reduced atmospheric humidity of 50 – 60%, what is the tea plant even bigger stressor than lack of soil moisture. Increasing the synthesis of carotenoids leads to a significant decrease in the ratio of the amount of chlorophyll to the number of carotenoids.

The identified patterns are common to all tea plants. However, the content of photosynthetic pigments also shows genotypic features (see Table 1).

The main photosynthetic pigment is chlorophyll-a **(Ognik et al., 2016; Potoroko et al., 2017; Platonova and Belous, 2019)**. Significant accumulation of chlorophyll in the leaves is characteristic of the control cv. Colchida. While the studied mutant forms contain chlorophyll a significantly lower (Table 1). The content of chlorophyll b indicates the level of adaptation of plants to low light **(Ognik et al., 2016; Potoroko et al., 2017; Steinman et.al., 2017; Platonova and Belous, 2019)**. For tea plants, this is not relevant, since the crop is grown in open spaces and pruning the trellis stimulates the growth of leaves on the upper part of it. But often a tightly closed trellis restricts the space open to sunlight, and many of the leaves of the side surfaces are in shadow. In this case, increased content of chlorophyll b is preferable for the photosynthetic activity of leaves of this tier **(Beneragama and Goto, 2010; Biswal et.al., 2012)**. We noted the same pattern as with chlorophyll a: more chlorophyll b accumulates in the control variety; and the differences are significant or at the border of materiality, as in the case of forms 3823 and 2264. Important is not only the content of pigments but also their ratio. In all the tea plants studied, the a-b ratio ranges from 2.81 mg. g^{-1} to 3.36 mg. g^{-1} and the differences between the forms are insignificant (Table 1).

The ratio of the amount of chlorophylls to carotenoids is a more informative sign since it indicates the degree of adaptation of plants to adverse conditions **(Thongsook and Barrett, 2005; Potoroko et al., 2017)**. The stability of the variety is higher than the ratio is lower. According to this indicator, all new forms of tea can be classified as fairly stable, in their flushes, the ratio of the amount of chlorophyll to carotenoids is significantly lower than in the cv. Colchida (see Table 1).

CONCLUSION

Thus, we determined the change in the activity of GPO and photosynthetic pigments in a freshly collected 3-leaf sprout. The presence of declines and peaks in the activity of the enzyme, which are associated with meteorological conditions of vegetation, is noted.

The existence of a close correlation between increased enzyme activity and precipitation is shown. The stressful period under hydrothermal conditions affected the functional state of plants.

The acute water deficit led to the manifestation of oxidative stress, which is expressed in an increase in the

activity of GPO, as a non-specific reaction to a stress factor.

The dynamics of GPO activity in a 3-leaf sprout is determined by the genotypic features of the variety: the lowest activity was observed in forms 3823 and 2264, which indicates a low intensity of redox reactions in stressful situations in these plants.

The participation of the pigment apparatus in the adaptation of the tea plant is directly related to the content of carotenoids. With a significant water deficit, there is a sharp increase in their synthesis. The ratio "chlorophylls/carotenoids" is an informative sign of the degree of adaptation of tea plants to adverse conditions. The revealed patterns are common to all tea plants.

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TRANSFER EFFICACY OF *ESCHERICHIA COLI* O157:H7 BETWEEN SURFACES OF GREEN MATURE TOMATOES AND COMMON FOOD PROCESSING MATERIALS

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ABSTRACT

The objectives of this study were: a) to evaluate *E. coli* O157:H7 survival on green mature tomatoes and squares of common food processing materials – stainless steel, plastic (HDPE), and vinyl conveyor belt (PVC) – post-drying, stored at 25 ºC in the humidified environment for four days; b) to determine pathogen transfer rates (wet, 90 minutes, or 24-hours drying post-inoculation), from inoculated tomato surfaces to uninoculated steel, plastic, and vinyl conveyor belt squares and conversely. It was shown that *E. coli* O157:H7 did not survive well on the surface of tomatoes, resulting in a decline from 5.3 $\log_{10}$ CFU.mL^{-1} 90 minutes post-drying to 1.4 $\log_{10}$ CFU.mL^{-1} on day 4. Similarly, the pathogen did not survive well on the surface of food processing squares, with numbers declining over 4 days from 4.04, 4.44, and 4.19 CFU.mL^{-1} of rinsate 90 minutes squares post-drying to 0.72, 0.50, 0.83 $\log_{10}$ CFU.mL^{-1}, which is close to the detection limit, for the steel, vinyl belt, and plastic, respectively. Successful cross-contamination between tomatoes and food processing surfaces was achieved during wet transfer; while transfer after 90 minutes inoculum post-drying and 24 hours were less successful. This can be explained by both lack of liquid media with suspended bacteria for transfer and fast pathogen die-off after desiccation. Dry transfers, as shown by the percentage of "positive" for pathogen presence tomatoes and squares, as well as bacterial counts, were more successful from tomatoes to squares, but not conversely. Special concern raised vinyl conveyor belt, where the surface picked up the most pathogen cells from the surface of tomatoes, resulting in 100% positive during 90 minute-dry transfers, followed by plastic (66.7% positive) and steel (55.6% positive). To summarize, we presented data on the possibility of cross-contamination between mature green tomatoes and common food processing surfaces, which may be interesting for the processors for risk evaluation.

Keywords: tomatoes; steel; vinyl conveyor belt; plastic; *E. coli* O157:H7; survival; transfer; cross-contamination

INTRODUCTION

Several investigations concluded that consumption of contaminated raw tomatoes can lead to enteric pathogen outbreaks, especially *Salmonella spp.* **(CDC, 2002; Cummings et al., 2001; Croby et al., 2005)**. There is significant consumption of raw tomatoes worldwide, for example, 42% of this commodity is eaten raw in the U.S., a total of 29.6 pounds of tomatoes consumed per person in 2016 alone **(USDA-ERS, 2016)**. **Beuchat and Ryu (1997)** noted that the sources of enteric pathogens on the tomatoes can be irrigation water, wash water, handling by workers, or contaminated surfaces. Various groups of bacteria exist on the surface of green mature tomatoes as it was shown by **Tokarskyy and Korda (2019a)**. This contamination can be removed by high-temperature treatment **(Tokarskyy et al., 2009)** or by gamma irradiation **(Schilling et al., 2009)**, which are often undesirable, creating processed, but not a fresh product. Gram-negative enteric pathogens will grow in the tomato at room temperature only if introduced into the flesh through abrasions, wounds, and stem scars **(Wei et al., 1995; Zhuang, Beuchat and Angulo, 1995; Daş, Gürakan and Bayindirli, 2006; Shi et al., 2007)**. Regarding the fate of the pathogens on the healthy tomato skin, it is believed that counts of Gram-negative enteric bacteria will decline over time, depending on tomato storage temperature, resuspension medium, and humidity **(Tokarskyy et al., 2018; Tokarskyy and Schneider, 2019)**. To the best of our knowledge, *E. coli* O157:H7 has not been implicated in tomato-related foodborne outbreaks, therefore, most research related to tomato safety was done with *Salmonella.* **Hirai (1991)** wrote that *Salmonella* spp. have always been regarded as having better survival rates after desiccation compared to *Escherichia coli*, therefore, data might be comparable at a first glance. For example, **Lang, Harris and Beuchat (2004)** showed that spot-inoculated tomatoes with *Salmonella* spp. or *E. coli* O157:H7 showed counts decline by 2.20 and 3.17 log, respectively, after twenty-four hours inoculum post-drying.

A review by **Kramer, Schwebke, and Kampf (2006)** suggested that lower temperatures, higher inocula, and the presence of protein, serum, or other organic matter favor the survival of bacteria on inanimate objects. **Hirai (1991)** showed that *E. coli* K12 resuspended in deionized water, inoculated on cotton lint and dried, died off upon desiccation (3.5 log reduction on day 1 and 4.5 log reduction on day 3), while resuspension in 2% Bovine Serum Albumin or 5% horse serum caused only ca. 2.0 log reduction on day 4. On the other hand, the humidity effect might be more complicated, as **Møretrø et al. (2010)** showed that shigatoxin-producing *E. coli* dried on plastic or steel had the highest inactivation rate at 85% RH, while survived the best at 98% and 70%. It can be argued that microorganisms in the dried up inoculum survive better at low humidity (no metabolic activity) compared to high humidity, where exhausted stationary culture, still metabolically active, slowly dies off. However, at low inoculation level and high organic matter high humidity might stimulate growth. **Møretrø et al. (2010)** showed that twelve Shiga-toxin producing *E. coli* strains, each analyzed separately, declined upon desiccation in Brain Heart Infusion broth (BHI) on the stainless steel (type 304) from 6 – 7 logs to 3 – 5 logs on day 1 and 2 – 3.5 logs on day 7. Follow-up studies comparing BHI and water, 12 °C and 20 °C, 70% RH, and 80% RH, showed a beneficial effect of BHI, 12 °C, and 70% RH for *E. coli* survival.

Mature green tomatoes contact with various food processing surfaces during processing steps, such as stainless steel (sorting tables), polyvinyl chloride surfaces (PVC, vinyl conveyor belts), high-density polyethylene (HDPE) parts of the various processing equipment. There is a possibility that a single tomato, highly contaminated with *E. coli* O157:H7, can transfer pathogen to other surfaces, while these surfaces, in turn, can contaminate hundreds of uncontaminated tomatoes. Therefore, the question of possible cross-contamination by *E. coli* O157:H7 between tomatoes and such food processing materials as stainless steel, HDPE, and PVC, remains open.

The first objective of the current study was to determine survival rates of *E. coli* O157:H7 on the surface of unwashed and undamaged green mature tomatoes, as well as on the surface of common packaging materials (plastic, HDPE; vinyl belt, PVC; and stainless steel) at summertime room temperature (25 ºC) within four days of storage. The second objective of the study was to investigate transfer rates of *E. coli* O157:H7 from the inoculated surface of tomatoes to the surface of these common food processing materials and vice versa, as influenced by the timing of the transfer.

Scientific hypothesis

We hypothesize that there is a potential of survival of *E.coli* O157:H7 on the surface of tomatoes and common food processing surfaces, and there is a significant possibility of pathogen transfer between surfaces not only when surfaces are wet, but also when they are dry.

MATERIALS AND METHODOLOGY

Rifampin preparation

0.4 grams of rifampin (Fisher Scientific, BP26795) was dissolved in 40 mL methanol (HPLC grade, Fisher Scientific), filter-sterilized (0.2 micron nylon filter, Fisher Scientific) to prepare sterile 10,000 ppm stock solution, and stored refrigerated (4 °C) in the darkness for no longer than a month. Rifampin stock solution was added to the cooled autoclaved Difco™ tryptic soy agar (TSA, Becton, Dickinson, and Co) or Bacto™ tryptic soy broth (TSB, Becton, Dickinson, and Co.) to yield 100 ppm final rifampin concentration, such as 0.1 mL rif stock to 10 mL TSB tube, or 10 mL rif stock to 1,000 mL TSA medium.

Tomato and food processing materials preparation

Green mature tomatoes variety Florida 47, unwashed and unwaxed, were acquired from local packinghouse (DiMare Company, Ruskin, Florida, U.S.A). Stainless steel squares (7.6 x 7.6 cm) were purchased from a local welding shop. Vinyl belt squares (7.6 x 7.6 cm, PVC-120, white polyester, one-side coated with PVC) were purchased from WL Deckert Co, Inc (Milwaukee, WI, U.S.A.). High-density polyethylene (HDPE, plastic) sheets (0.16 cm thick) were purchased from US Plastic Corp (Lima, OH, U.S.A.) and manually cut into 7.6 x 7.6 cm squares. All squares were run through the Lancer dishwasher (Lancer USA, Longwood, FL, U.S.A.), and manually rinsed twice with deionized water before drying. Therefore, food processing surfaces were classified as used. Stainless steel and vinyl belt squares were also reused after autoclaving.

Escherichia coli O157:H7 culture preparation

Two rifampin-resistant (200 ppm) strains of *Escherichia coli* O157:H7 (MDD20, MDD326) and two rifampin-sensitive strains (MDD19 and MDD 327NA), were kindly provided by Dr. Michelle Danyluk's lab from the University of Florida. ATCC 35150 rifampin-sensitive strain was acquired from American Type Culture Collection (Manassas, WI, U.S.A.). All three rifampin-sensitive strains were mutated to acquire rifampin resistance by transferring a pure culture from TSA plates (37 °C, 24 hours) to 10 mL TSB-rif 5ppm broth (37 °C, 24 hours), followed by sequential transfer of 0.1 mL aliquot to TBS containing 10, 20, and 40 ppm rifampin. Final turbid cultures (40 ppm rifampin) were streaked on TSA-rif200 plates (37 °C, 24 hours), and a single colony was transferred to TSB-rif200 broth to confirm growth. Five rif-resistant *E. coli* O157-H7 strains were maintained on TSA-rif80 ppm slants at 4 °C with bi-weekly transfers to fresh TSA-rif80 slants.

For each replication of the experiments, five strains were streaked on TSA-rif100 plates (37 °C, 24 hours), followed by three consecutive one-loopful transfers to 10 mL TSB-rif100 tubes (37 °C, 12 hours, 12 hours, and 18 hours). A pathogenic cocktail (10 mL, 10^9 CFU.mL^{-1}) was prepared by mixing 2 mL of each culture from the third broth. The cocktail was centrifuged (4,300 g, 10 minutes, Sorvall RC-5B centrifuge, DuPont Instruments) and washed once in 10 mL Dulbecco A phosphate-buffered saline (PBS, Oxoid, Hampshire, England), followed by

final centrifugation (4,300 g, 10 minutes) and resuspension in 10 mL 0.1% peptone (Bacto peptone, Becton Dickinson and Co, Sparks, USA). Inoculum concentration was confirmed by serial dilutions in Buffered Peptone Water (BPW, Becton, Dickinson, and Co.) and pour plating using TSA-rif100.

Tomato inoculation and storage experiment

Fifteen mature green tomatoes were inoculated with 0.1 mL of the pathogenic cocktail as 10 spots of equal size around the blossom end each (10^8 CFU.tomato^{-1}). One set of four tomatoes plus one tomato for immediate sampling was left uninoculated and served as negative controls. The procedure was carried out in a biosafety hood and tomatoes were allowed to dry for 90 minutes before moving into a 25 °C incubator. A shallow pan with water was placed inside the incubator to humidify the environment, while temperature and humidity were recorded for four days with 10-minute sampling intervals (Hobo® U12 data logger, Onset Computer Corp, Pocasset, MA). Sets of three inoculated and dried tomatoes with one negative control tomato were tested immediately after drying in the biosafety hood (day 0). Other tomatoes were sampled on day 1, day 2, day 3, and day 4 from a 25 °C incubator.

Food processing surfaces inoculation and storage experiment

Squares (7.6 cm by 7.6 cm) of the described earlier materials were spot inoculated with 0.03 mL of 10^9 CFU.mL^{-1} five strains rif-resistant *E. coli* O157:H7 cocktail in 0.1% peptone. The inoculum was allowed to dry for 90 minutes in the biosafety hood before squares were moved into a 25 ºC incubator. A shallow pan with water was placed in the incubator to humidify the environment and temperature/humidity were monitored for four days as described previously. Sets of three inoculated plus one negative control squares of each type were plated immediately after drying (day 0), as well as on day 1, day 2, day 3, and day 4.

Tomato and squares inoculation for the transfer studies

Two separate studies, involving transfers from tomatoes to food processing materials surfaces and vice versa, were performed. Mature green tomatoes were surface inoculated on a healthy circle-marked spot with a single 30 µL drop of pathogenic bacterial cocktail inside biosafety hood (3 x 10^7 CFU.tomato^{-1}). Two sets of three steel, vinyl belt, or HDPE squares were firmly pressed against tomato surface for one second (one square per each tomato) either immediately (wet transfer), 90 minutes after the inoculum has dried up on the surface, or 24 hours after tomato inoculation. The first set of wet transfer was analyzed immediately (W, day 0), while the second set of squares was placed under the biosafety hood to allow transferred liquid to dry on squares for 90 minutes. The second set was then moved to a 25 °C incubator and analyzed 22.5 hours later (W, day 1). Similarly, one set of 90 minutes dry transfer squares (90 min dry, day 0) was analyzed immediately and another set was placed in a 25 °C incubator and tested for *E. coli* 22.5 hours later (90 min dry, day 1). The last set of tomatoes was placed for an additional 22.5 hours incubation at 25 °C following 90 minutes drying period inside biosafety hood before two sets of steel, vinyl, and HDPE squares were pressed against inoculated spots and analyzed for pathogen transfer efficiency either immediately (24 h dry, day 0), or 24 hours later (24 h dry, day 1) after storage in the same incubator (25 °C). The shallow container filled with water was placed inside a 25 °C incubator for the duration of the study to humidify the atmosphere and temperature/humidity was monitored as described previously with Hobo® U12 data logger.

On each of three days, a set of negative control squares (one of each) was touched to the marked surface of uninoculated tomatoes and analyzed as a negative control to ensure the absence of rif-resistant microflora on tomatoes and squares.

Transfers from squares to tomato surfaces were done as described previously but in the opposite direction of inoculation and transfer.

Escherichia coli O157:H7 recovery from tomatoes and squares

To recover pathogen, a single tomato or a square was transferred to 20 mL BPW in a stomacher bag and vigorously manually shaken for 30 seconds, rubbed for 30 seconds, and shake again for 30 seconds. The rinsate was plated directly or serially diluted in 9 mL BPW tubes before plating using the pour plate method and TSA-rif100 medium. The plates were incubated for 24 hours at 37 ºC before counting.

Statistic analysis

Escherichia coli O157:H7 survival on tomatoes (three replications) and the squares (four replications) results were analyzed separately using one-way ANOVA (factor "day": day 0; day 1; day 2; day 3; day 4) with means separated using Fisher LSD procedure. Transfer studies were repeated three times and count data were analyzed using one-way ANOVA and treatment factor (W day 0; W day 1; 90 min dry day 0; 90 min dry day 1; 24 h dry day 0; 24 h dry day 1) labeled by each material (steel, belt, plastic). Samples yielding no counts were assigned a limit of detection count. Percent positive samples were calculated for transfer studies as well, for each treatment for each square, combining data from three replications. Statistical analysis was done using commercially available software Statistica ver.10.0 (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

For tomato surface survival studies, *E. coli* O157:H7 numbers declined 1.4 log units from theoretical inoculation level of 6.8 log units per mL of rinsate to 5.3 logs upon 90-minute drying, and continued to decline significantly and rapidly during storage at 25 ºC (p <0.05), resulting in final counts of 1.5 logs on day 4 (Figure 2). Similarly, **Lang, Harris and Beuchat (2004)** showed that *E. coli* O157:H7 counts in 5% horse serum on the dried spot-inoculated tomatoes decreased 1.07 logs after 1-hour drying and 3.17 logs 24 hours post-drying from initial 7.22 $\log_{10}$ CFU.tomato^{-1}.

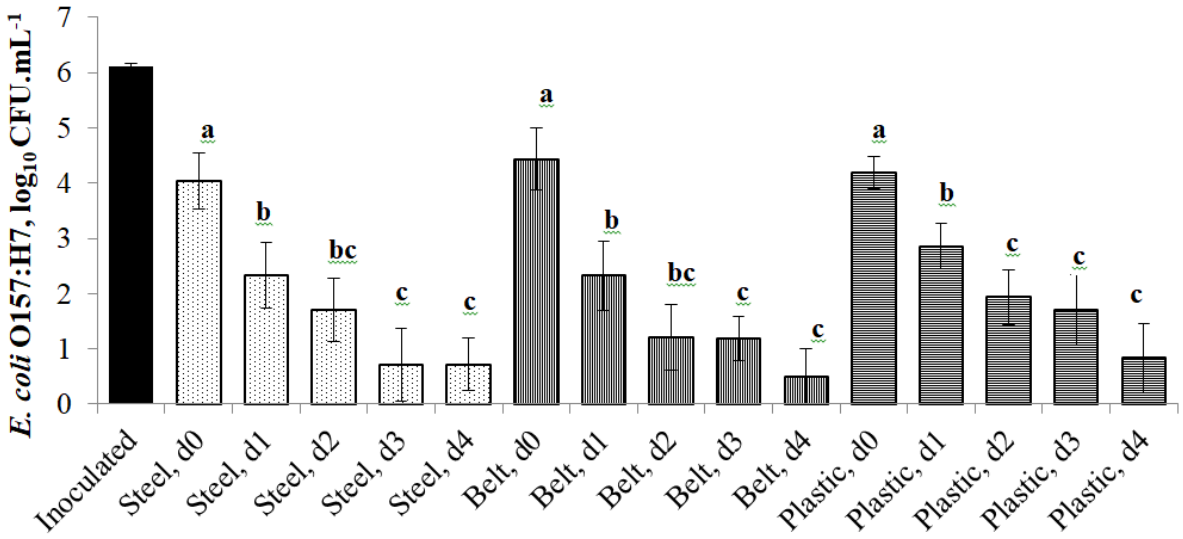

Figure 1 Recovery of *E. coli* O157:H7 from squares either immediately after drying (d0), or after storage for four days (d0-d4) at 25 ºC. Note: Counts expressed as log10 CFU.mL^{-1} recovered from 20 mL rinsate. Inoculated level calculated theoretically based on stationary culture and is shown for reference. The same letters within the same material (steel, belt (PVC), or plastic (HDPE)) mean no significant difference (p >0.05). *Average air relative humidity ±SD: replication No 1 = 67.4 ±2.2%; replication No 2 = 70.8 ±2.0%, replication No 3 = 71.6 ±2.1%, replication No 4 = 73.2 ±1.9%.

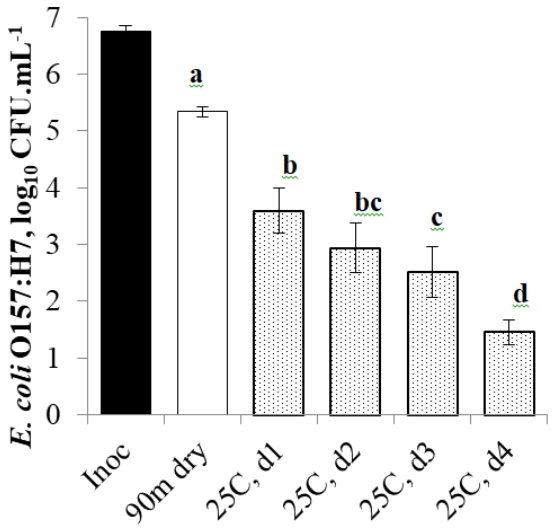

Figure 2 Recovery of *E. coli* O157:H7 from inoculated tomatoes either immediately after drying (90 min dry), or after storage for four days (d1-d4) at 25 ºC. Note: Counts expressed as $\log_{10}$ CFU.mL^{-1} recovered from 20 mL rinsate. Inoculated level calculated theoretically based on stationary culture concentration and is shown for reference. The same letters mean no significant difference (p >0.05). *Average air relative humidity ±SD: replication No 1 = 58.8 ±3.6%, replication No 2 = 59.4 ±3.8%, replication No 3 = 59.5 ±4.1%.

Table 1 Percentage of squares and tomatoes yielding at least 1 CFU.mL^{-1} of *E. coli* O157:H7 in rinsate after inoculated tomatoes touched squares or inculated squares touched tomatoes. Cross-contaminated items were checked for *E. coli* either immediately after the transfer (d0), or stored 24 h after the transfer at 25 ºC (d1).

	W P d0	**W B d0**	**W S d0**	**90m P d0**	**90m B d0**	**90m S d0**	**24h P d0**	**24h B d0**	**24h S d0**	**W P d1**	**W B d1**	**W S d1**	**90m P d1**	**90m B d1**	**90m S d1**	**24h P d1**	**24h B d1**
S2T	100	100	100	11.1	44.4	11.1	0	0	0	88.9	100	88.9	11.1	0	0	0	0
T2S	100	100	100	66.7	100	55.6	11.1	33.1	0	100	100	100	0	100	0	11.1	22.2

Note: W – wet; 90 m – 90min dry; 24 h – 24 h dry. P – plastic; B – belt; and S – steel. *T2S – Tomatoes to Squares transfer; S2T – Squares to Tomatoes transfer.

Similar results were obtained by **Tokarskyy and Korda (2019b)** for surface inoculated tomatoes with *E. coli* O157:H7 and stored for four days in a high humidity incubator at 25 °C. They noted better survival of pathogen if final inoculum was prepared in less hygroscopic 0.1% diluted peptone water, compared to buffered peptone water, where pathogen declined from 5.4 $\log_{10}$ CFU.mL^{-1} in 90 min dry tomatoes to 1.4 $\log_{10}$ CFU.mL^{-1} on those stored for four days at 25 °C **(Tokarskyy and Korda, 2019b)**. The decrease in numbers of viable *E. coli* O157:H7 on the surface of bruised and unbruised tomatoes at 20 °C was even more drastic when a low contamination level was used (4.0 $\log_{10}$ CFU.tomato^{-1}), where counts dropped to below detection level in just three days **(Tokarskyy et al., 2018)**.

To summarize, *E. coli* O157:H7 did not survive well on the intact surface of tomatoes at 25 °C.

Survival of *E. coli* O157:H7 on the surface of common food processing materials is shown in Figure 1. The counts declined from theoretical inoculation level of 6.1 $\log_{10}$ CFU.mL^{-1} as calculated per mL of the rinsate to 4.0, 4.4, and 4.2 $\log_{10}$ CFU.mL^{-1} for steel, belt, and plastic, respectively, upon drying (p <0.05). After four days incubation period at 25 °C, the counts on average felt below 1.0 $\log_{10}$ CFU.mL^{-1} for all squares. These data are similar to **Kusumaningrum et al. (2003)**, who showed that *Salmonella enteritidis* was recovered from inoculated steel squares after drying for at least 4 days at a high contamination level (10^5 CFU.cm^{-2}), while at a moderate level (10^3 CFU.cm^{-2}) and low level (10 CFU.cm^{-2}) inoculation counts went below detection limit within 24 hours and 1 hour, respectively. **Møretrø et al. (2010)** showed that STEC *E. coli* inoculated on stainless steel in water or BHI and dried declined by ca 1.6 and 3.6 logs, respectively, at 20 °C after 24 hours post-drying. The authors noted that results for polyoxymethene copolymer were not significantly different from stainless steel **(Møretrø et al., 2010)**. It might be noted that food-grade (type 304) stainless steel was used for the study, as different metal alloys might impact survival. As was shown by **Jiang and Doyle (1999)**, *E. coli* O157:H7 inoculated in 0.1% peptone and dried on glass and coins at 4.7 $\log_{10}$ CFU.coin^{-1} declined at room temperature to below detection level on day 4, 7, 9, 11, and 11 for glass, pennies, nickels, dimes, and quarters, respectively. Contrary, **Tokarskyy and Korda (2019b)** showed better survival of *E.coli* O157:H7 on the surface of unwaxed cardboard, with counts declining from 4.5 to only 2.5 $\log_{10}$ CFU.mL^{-1} after 4-day storage at 25 °C, what can be attributed to the porous and water-absorbing nature of cardboard surface.

To summarize, little to no potential of *E. coli* O157:H7 survival was shown in the current study for common impervious food processing surfaces.

Several research groups used different approaches and techniques to measure transfer rates of enteric pathogens from inanimate surfaces to produce and conversely **(Buchholz et al., 2012; Soares et al., 2012; Brar and Danyluk, 2013; Jensen et al., 2013; Todd-Searle et al., 2020).** Going further, **Buchholz et al. (2012)** even used pilot pant settings for transfer studies and showed that *E. coli* 0157:H7 continuously cross-contaminated lettuce through flume tank, conveyor tank, and shredder, considering that all surfaces remained wet through the processing line and sanitizer was not applied. **Brar and Danyluk (2013)** studied Salmonella transfer from contaminated plastic gloves to tomatoes and conversely, after 24 hours inoculum drying on the surfaces. They did not find any difference between transfers from dirty and from clean reusable gloves (**Brar and Danyluk, 2013)**.

Results of our transfer studies were expressed either as percent positive (where at least one typical *E. coli* O157:H7 CFU per 1 mL of rinsate was detected) or as counts, total $\log_{10}$ CFU.item^{-1} (either a food processing surface square or a tomato), and are shown in Table 1 and Figure 3 and Figure 4. Samples yielding no counts were assigned a limit of detection count. Overall, wet transfers (W) yielded the most consistent data, with 100% transfers being positive on both day 0 and day 1 (Table 1). Dry transfers (90 min dry and 24 h dry) appeared to be less efficient, partially because of lack of wetness with liquid carrying bacteria and having adhesive properties, and possibly due to the pathogen number decline during drying and storage. **Burnett, Chen, and Beuchat (2000)** hypothesized that *E. coli* O157:H7 may firmly attach to the surfaces of fruits as evidenced by confocal scanning laser microscopy and may evade decontamination and detachment, which could be one of the explanations why dry transfer was not efficient in our study. However, dry transfers from tomatoes to squares appeared to be more efficient than vice versa (Table 1, Figure 3 and Figure 4), possibly due to the hydrophobic properties of the tomato peel surface. Interestingly, the vinyl belt picked up the most pathogen cells from the surface of tomatoes, resulting in 100% positive during 90 min dry transfers, followed by plastic (66.7% positive) and steel (55.6% positive). Subjectively, the vinyl belt appeared to have a "sticky" surface. Similarly, dry transfers at 24 h storage were the most efficient from tomatoes to the vinyl belt, followed by plastic (Figure 3). Similar results were shown by **Tokarskyy and Korda (2019b),** who showed that wet transfer of *E.coli* O157:H7 was more efficient between tomatoes and cardboard comparing to dried surfaces, as well as transfer from tomatoes to cardboard was more efficient than vice versa. **Todd-Searle et al. (2020)** showed that the transfer of Salmonella between tomatoes and plastic mulch or soil was dependent on the dryness of the inoculum, contact time, and contact surface. They also noted that transfer from plastic mulch was greater than from soil, possibly due to the surface characteristics, while wet and 1-hour dry transfers were more efficient than 24-hours dry transfers **(Todd-Searle et al., 2020)**. **Soares et al. (2012)** showed that *Salmonella* spp. easily transferred from wet poultry skin to the cutting surfaces made of wood, stainless steel, and plastic (100% positive for contamination), and then from those contaminated surfaces to the red tomatoes (also 100% tomatoes became contaminated), unless cutting surfaces were washed with soap followed by surface sanitation. **Jensen et al. (2013)** noted that freshly inoculated lettuce or celery transferred more bacteria (ca. 2 % to ca. 25 % of the inoculum) comparing with freshly inoculated carrots or watermelon (ca. 1% to 8%) to the surfaces made of ceramic, stainless steel, glass, and plastic. Such high transfer rates were probably due to the residual moisture left after fresh inoculum application.

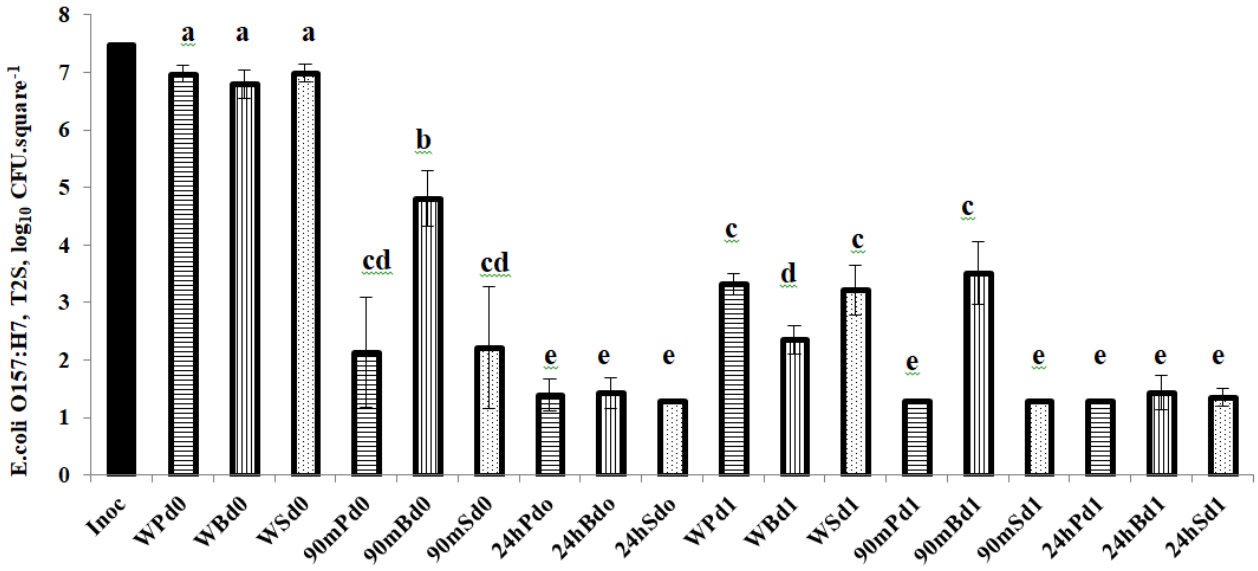

Figure 3 Total *E. coli* O157:H7 counts per square after pathogen transfer from tomato compared to total inoculated log_{10} CFU.tomato^{-1} (Inoc). Note: Tomatoes (W – wet; 90 m – 90 min dry; 24h – 24 h dry) touched squares (P, plastic; B, belt; and S, steel) which were analyzed either immediately (d0) or 24 hours later (d1). Detection limit 1.3 log per item. The same letters mean no significant difference (p >0.05). Tomato inoculation level calculated theoretically based on inoculum concentration and is shown for reference. T2S – Tomatoes to Squares transfer. *Average air relative humidity ±SD: replication No 1 = 61.1 ±9.0%, replication No 2 = 68.3 ±8.1%, replication No 3 = 66.2 ±6.2%.

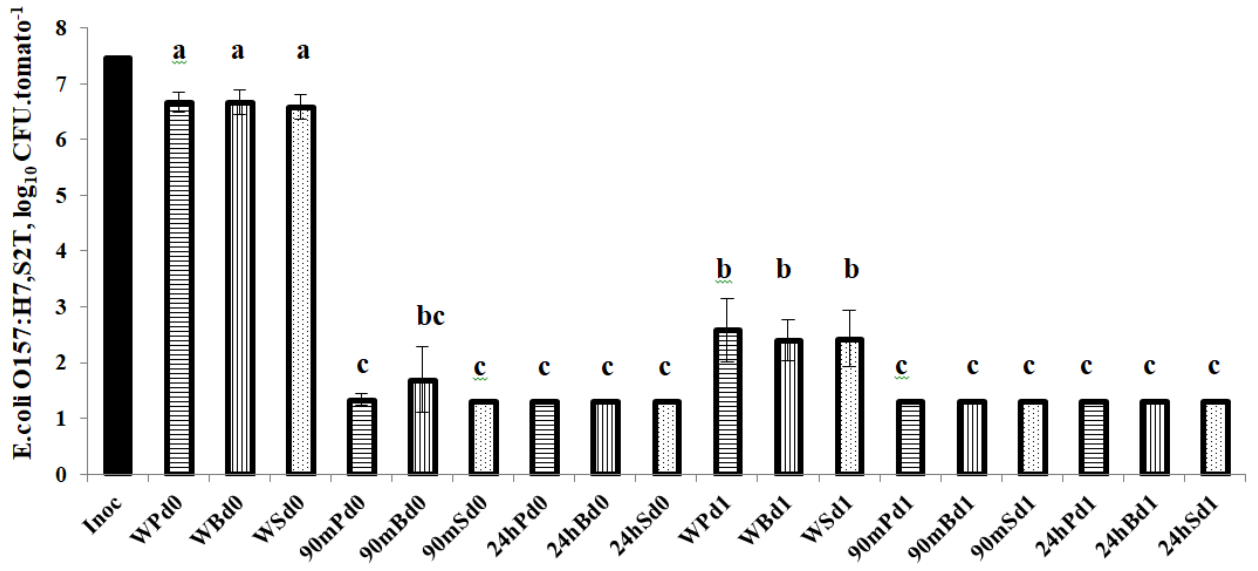

Figure 4 Total *E. coli* O157:H7 counts per tomato after transfer from inoculated squares. Note: Squares (W – wet; 90 m – 90 min dry; 24 h – 24 h dry) of different types (P, plastic; B, belt; and S, steel) touched tomatoes which were analyzed either immediately (d0) or 24 hours later (d1). Detection limit:1.3 log10 CFU per item. The same letters mean no significant difference (p >0.05). Square inoculation level (Inoc) calculated theoretically based on inoculum concentration and is shown for reference. S2T – Squares To Tomatoes transfer. *Average air relative humidity ±SD: replication No 1 = 62.9 ±6.7%, replication No 2 = 70.2 ±7.0%, replication No 3 = 70.4 ±6.3%.

However, after one hour of drying time, the transfer rate from inoculated celery, carrot, and lettuce decreased significantly to less than 0.01 to ca. 5% and to less than 1% to ca. 5% for watermelon **(Jensen et al., 2013).** The authors concluded that the surface moisture and the direction of the transfer had the greatest influence on microbial transfer rates **(Jensen et al., 2013).** To support this statement, **Todd-Searle et al. (2020)** also emphasized that tomatoes should be harvested dry, not wet, to avoid cross-contamination.

To summarize, the dry transfer is limited and is more efficient from the tomatoes to the packaging squares, and less efficient from packaging squares to tomatoes. Speaking of risks involved, the vinyl belt appeared to be the most affected. The study has limits in accuracy, as chances of getting 1 CFU.plate^{-1} are not statistically profound.

CONCLUSION

Pathogen transfers are of great concern if the surface is wet, but less of a concern if the surface is dry. Because pathogens do not survive well under the conditions tested, prolonged storage reduces the chances of cross-contamination. Dry transfers from tomatoes to food contact surfaces are more efficient compared to transfers from food contact surfaces to tomatoes. This could be due to the hydrophobic nature of the tomato surface. The results suggest that the vinyl belt (PVC) might represent a higher risk. Overall, we partially failed our hypothesis, showing that there is a low possibility of pathogen transfer if surfaces are dry after prolonged tomato storage under proposed model conditions.

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EFFECT OF SOMATIC CELL COUNT ON MILKABILITY AND MILK COMPOSITION OF EWES

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ABSTRACT
The trial aimed to study the effect of somatic cell count, breed, milk flow type, and parity on the milkability and milk composition of ewes. The flock consists of purebred Lacaune ewes (LC; n = 29) and crossbreds ewes of Improved Valachian (IV x LC; n = 35) and Tsigai (TS x LC; n = 37) with LC (with a genetic portion of Lacaune 25 and 50%). Ewes were assigned according to somatic cell count (SCC) to one of the following three groups: SCC ≤300,000 cells per mL (SCC Group 300,000), SCC between 300,000 and 1000,000 cells per mL (SCC Group 300,000 – 1000,000), SCC ≥1000,000 cells per mL (SCC Group 1000,000). 56% of evaluated ewes had less than 300,000 cells per mL and 29% more than 1000,000 cells per mL in milk. No significant differences were observed between different groups of SCC in total, machine milk yield, and the proportion of milk yield in 30 s and 60 s. The significant differences were observed between SCC Group 300,000 and SCC Group 300,000 – 1000,000 in the proportion of machine stripping from total milk yield (41 ±2 vs. 57 ±4%). Milk flow type had a significant effect on all evaluated milkability parameters but not on milk composition. Ewes on fourth parity had the highest proportion of machine stripping from total milk yield then ewes on third, fifth, and sixth and higher (60% vs. 47, 45, 46%; resp.). Effect of SCC Group on milk composition manifested only in solids not fat. The significant differences were observed between SCC Group 1000,000 to SCC Group 300,000 and SCC Group 300,000 – 1000,000 (10.75 ±0.08% vs. 11.05 ±0.06 and 11.15 ±0.11%, $p \leq 0.0004$).

Keywords: ewe; somatic cell count; milk composition; SCC

INTRODUCTION
In ewes and other dairy animals, somatic cells are an important natural component of milk. Their number is used as an indirect predictor of udder health status and milk quality as they are involved in the protection of the mammary gland from infection as part of the innate immune system **(Rupp and Boichard, 2000; Guzel et al., 2017; Tančin et al., 2006; Tančin et al., 2007; Tančin, Ipema and Hogewerf, 2007)**. Somatic cell count (SCC) in milk is influenced by several factors: animal species, production level, physiological processes (such as estrus or stage of lactation), animal individuality, and also environmental factors and farm management **(Rupp and Boichard, 2000; Tančin et al., 2016; Tančin et al., 2017; Paape et al., 2007; Margetín et al., 2013)**. According to **Ranucci and Morgante (1996)** and **Bergonier et al. (2003)**, somatic cells in healthy ewe milk consist of macrophages, polymorphonuclear neutrophil leukocytes (which have an important biological function of phagocytic activity), lymphocytes, and, to a lesser extent other cell types (eosinophils, epithelial cells, and unidentifiable cells). As in dairy cows, also in goats or sheep, an increase in SCC has been reported as a consequence of infection **(Poutrel et al., 1997; Paape et al., 2001; Paape et al., 2007; Bergonier and Berthelot, 2003; Bergonier et al., 2003; Luengo et al., 2004; Moroni et al., 2005; Koop et al., 2010)**. During mastitis, milk SCC increases mainly as a result of increased migration of leukocytes from blood to mammary tissue **(Leitner et al., 2003; Le Roux, Laurent, and Moussaoui, 2003)**. Because there is a strong relationship between udder health and the amount of somatic cells in milk, limits have been set for SCC in milk in many countries. These limits determine which milk can be on the market or what penalties in the contractual terms of payment are imposed by the milk producer when the milk does not meet the required criteria **(Berger et al., 2004; Haenlein, 2002; Kalantzopoulos et al., 2004; Raynal-Ljutovac, Gaborit and Lauret, 2005)**. However, there are no limits to SCC in sheep's milk as it is in cow's milk in Slovakia **(Tančin et al., 2017)**. The major income from dairy animals is derived from milk; therefore, factors that reduce milk quantity and quality can cause high economic losses to the farmers **(Sutera et al., 2018)**.

The trial aimed to study the effect of SCC on the milkability and milk composition of ewes. Possible effects of breed, milk flow type, and parity were evaluated too.

Scientific hypothesis

In this study, we hypothesized that ewes, which had lower SCC than 300,000 mL^{-1} (SCC Group 300,000), would have better parameters of milkability and milk composition than ewes with the higher SCC. The second hypothesis was that ewes with two peaks and plateau milk flow types would have a higher milk production and milk fat content than ewes with one peak and plateau II. The third hypothesis was that breed affects the production parameters. The fourth hypothesis was that parity affects the production parameters and SCC.

MATERIAL AND METHODOLOGY

Animal and experimental design

The study was carried out in June in the flock of 101 mid-lactated ewes (102 ±5 days in lactation) their 3rd – 9th parity at one evening milking. The flock consists of purebred Lacaune ewes (LC; n = 29) and crossbreds ewes of Improved Valachian (IV x LC; 35) and Tsigai (TS x LC; 37) with LC (with a genetic portion of Lacaune 25 and 50%). The ewes were milked in a one-platform milking parlour with 24 stalls and one milking unit per 2 milking stalls. The milking machine was set to provide 160 pulsations per minute in a 50:50 ratio and a vacuum level of 39 kPa. During each milking, ewes received 0.1 kg concentrate per head in the parlour. Ewes were milked routinely twice daily at 8:00 and 20:00 without any udder preparation. At the end of milking, machine stripping was performed (machine stripping started when milk flow rate declined to 0 $L.min^{-1}$ but not earlier than 70 s from the beginning of milking). Short manual udder massage was performed by machine stripping.

Milk flow recording and samples analysis

Milk flow kinetic was recorded using an electronic jar that collected the milk during the next three consecutive evening milkings. Within the jar, there was a 2-wire compact magnetostrictive level transmitter (NIVO-TRACK, NIVELKO Ipari Elektronika Rt, Budapest Hungary) connected to a computer. The milk level was continuously measured by a transmitter that recorded the position of the float in the jar on a computer once per second. The milk flow patterns were drawn by using a formula by **Mačuhová et al. (2008)**. The following parameters of milkability were evaluated: total milk yield (L), machine milk yield (L), machine stripping yield (L), machine stripping yield from total milk yield (%), milking time (i.e. time from attaching of clusters until the milk flow ceased before stripping; s), milk yield in 30 s (L), and milk yield 60 s (L).

According to SCC, ewes were assigned to one of the following three groups: SCC ≤300,000 cells per mL (SCC Group 300,000), SCC between 300,000 and 1000,000 cells per mL (SCC Group 300,000 – 1000,000), SCC ≥1000,000 cells per mL (SCC Group 1000,000).

Milk flow curves were evaluated according to **Marnet, Negrao and Labussière (1998)**, **Rovai et al. (2002)** and **Mačuhová et al. (2008)** into 4 milk flow types; 1 peak (1P; without notable milk flow after 40 s of milking), 2 peaks (2P; two separate milk emissions), plateau (PL; milk flow with longer duration of steady phase and milk flow rate during plateau phase >0.4 $L.min^{-1}$ at least for 20 s), and plateau low (PLII; milk flow curves with steady milk flow during milking for 20 s but with milk flow rate ≤0.4 $L.min^{-1}$ or >0.4 $L.min^{-1}$ shorter than for 20 s at plateau phase). In 1 animal no milk flow occurred, and the curve of milk flow type could not be identified.

Individual milk samples were collected after milking from the jar for composition analysis. Milk composition was analyzed for the percentage of fat, protein, lactose, solids, and solids-not-fat with MilkoScan FT120 (Foss, Hillerød, Denmark). SCC was analyzed with Somacount 150 analyzer (Bentley Instruments, lnc, Chaska, Minnesota).

Statistical analysis

The data set consisted of 101 measurements belonging to 101 ewes. Mixed model (Mixed procedure; SAS/STAT 9.1, 2002 – 2003) was applied to study the influence of the sources of variation in studied traits (parameters of milkability and milk composition).

$$y_{ijkl} = \mu + \text{SCC GROUP}_i + \text{FLOW}_j + \text{BREED}_k + \text{PARITY}_l + e_{ijkl}$$

where: y_{ijkl} – individual observations of studied parameters of milkability and milk composition, μ = overall mean, SCC GROUP_i = fixed effect of SCC group (i = 1 to 3; ≤300,000, between 300,000 and 1000,000, ≥1000,000 cells per mL), FLOW_j = fixed effect of milk flow type (j = 1 to 4; 2P, 1P, PL, PLII) + BREED_k = fixed effect of Breed (k = 1 to 3; TS x LC, LC, IV x LC) + PARITY_l = fixed effect of parity (l = 1 to 4; 3, 4, 5, ≥6), e_{ijkl} = random error, assuming $e$ijkl ~ N(0, I σ_e^2).

The fixed effects of the model were estimated using the LSM (Least Squares Means) method. Statistical significance was tested by Fischer's F-test and differences between the estimated levels of effects were tested by Scheffe's multiple range tests.

RESULTS AND DISCUSSION

In Table 1, there are presented basic statistics of studied traits and in Table 2, *p*–values for the statistical significance of tested factors on evaluated parameters. SCC has been described in numerous studies as a useful method for diagnosing intramammary infection in monitoring udder health. In this study, animals were classified according to SCC in three groups (SCC Group ≤300,000 cells per mL; SCC Group between 300,000 and 1000,000 cells per mL; SCC Group ≥1000,000 cells per mL). 56% of evaluated ewes had SCC lower than 300,000 cells per mL and 29% more than 1000,000 cells per mL. SCC Group had no significant effect on parameters of milkability (except machine stripping yield from total milk yield; Table 3) or parameters of milk composition (except for solids not fat (%)) (Table 5). There is a discussion on the SCC threshold level for diagnostic purposes **(Raynal-Ljutovac et al., 2007; Albenzio et al., 2012; Rovai et al., 2015; Tvarožková et al., 2019)**.

the SCC over 600,000 cells per mL is considered as high.

Table 1 Characteristics of statistical file of studied traits.

Label	N	Minimum	Maximum	Mean	Std Error
Total milk yield (TMY), L	101	0.112	1.001	0.392	0.017
Machine milk yield (MMY), L	101	0.067	0.781	0.25	0.015
Milking time, s	101	15	98	50	2
Milk flow latency, s	101	2	78	18	1
Milk yield in 30 s, L	101	0	0.399	0.127	0.008
Milk yield in60 s, L	101	0.024	0.781	0.224	0.014
Machine stripping yield/TMY, %	101	5.22	84.56	37.4	1.59
log SCC	101	4.908	7.874	6.473	0.064
Fat, %	101	4.65	8.9	6.23	0.08
Protein, %	101	4.35	6.61	5.27	0.04
Lactose, %	101	4.4	5.18	4.87	0.02
Solids, %	101	14.72	19.97	16.99	0.1
Solids not fat, %	101	10.12	12.23	10.96	0.037

Table 2 Statistical significance (*p*-values) of tested factors on evaluated parameters.

	Total milk yield (TMY), L	Machine milk yield, L	Milking time, s	Milk flow latency, s	Milk yield in 30 s, L	Milk yield in 60 s, L	Machine stripping yield/TMY, %	Fat, %	Lactose, %	Solids, %	Solids not fat, %
SCC	0.7795	0.4299	0.0713	0.2366	0.0644	0.1418	0.0013	0.2565	0.1318	0.4442	0.0004
Milk flow type	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.3524	0.9828	0.2968	0.1011
Breed	0.051	0.0025	0.0014	0.605	0.0002	0.0002	0.0188	0.3962	0.0042	0.1313	0.0271
Parity	0.9251	0.8362	0.2847	0.0963	0.4726	0.6640	0.0124	0.0578	0.5411	0.0373	0.0151

Table 3 Parameters of milkability according to Somatic cell cound (SCC) and milk flow type.

	SCC Group			Milk flow type			
Parameters	**≤300,000**	**300,000 – 1000,000**	**≥1000,000**	**2P**	**1P**	**PL**	**PL II**
N	57	15	29	37	27	23	13
Total milk yield (TMY), L	0.363 ±0.027	0.399 ±0.050	0.367 ±0.036	0.416 ±0.032^{a}	0.289 ±0.032^{b}	0.541 ±0.038^{a}	0.367 ±0.048^{b}
Machine milk yield, L	0.212 ±0.022	0.159 ±0.039	0.196 ±0.029	0.250 ±0.025^{a}	0.164 ±0.025^{a}	0.382 ±0.030^{b}	0.152 ±0.038^{a}
Milking time, s	50 ±3	59 ±5	47 ±3	67 ±3^{ac}	29 ±3^{b}	54 ±4^{a}	66 ±4^{c}
Milk flow latency, s	23 ±2	20 ±3	26 ±2	13 ±2^{a}	11 ±2^{a}	18 ±2^{a}	34 ±3^{b}
Milk yield in 30 s, L	0.105 ±0.014	0.045 ±0.025	0.081 ±0.018	0.099 ±0.016^{ab}	0.144 ±0.016^{a}	0.154 ±0.019^{a}	0.0303 ±0.024^{b}
Milk yield in 60 s, L	0.187 ±0.020	0.112 ±0.037	0.165 ±0.027	0.208 ±0.023^{a}	0.151 ±0.023^{a}	0.356 ±0.028^{b}	0.114 ±0.036^{a}
Machine stripping/TMY, %	41.27 ±2.43^{a}	57.23 ±4.43^{b}	49.92 ±3.22^{b}	39.92 ±2.81^{ab}	42.52 ±2.77^{ab}	31.17 ±3.41^{a}	55.30 ±4.30^{b}
log SCC	-	-	-	6.297 ±0.111	6.666 ±0.125	6.346 ±0.148	6.827 ±0.186

Note: ^{a,b} The means in the same line without same letter were significantly different at $p \leq 0.05$.

However, in most studies, 300,000 or even 400,000 cells per mL are considered as normal values in sheep's and goat's milk **(Kern et al., 2013; Tančin, 2017; Oravcová, Mačuhová and Tančin, 2018)**. On the other hand, whereas **Kern et al. (2013)** indicate already 400,000 cells per mL in meat breeds and 300,000 cells per mL in dairy breeds as a threshold level to assist the farmer in detecting of the udder health problems, in the studies of **Tančin et al. (2017)** and **Oravcová, Mačuhová and Tančin (2018)**, Only by 1000,000 cells per mL is milk considered as mastitis milk **(Tančin et al., 2017)** or ewe as infected **(Berthelot et al., 2006)**.

The significant differences were observed between SCC Group ≤300,000 and SCC Group 300 – 1000,000 in the proportion of machine stripping from total milk yield (41 ±2 vs. 57 ±4%). The tendency of the longest milking time was observed in SCC Group 300,000 and 1000,000 in comparison to SCC Group ≥1000,000 (59 vs. 47 s).

Table 4 Parameters of milkability according to breed, and parity.

	Breed			Parity			
Parameters	**TS x LC**	**LC**	**IV x LC**	**3**	**4**	**5**	**≥ 6**
N	37	29	35	29	20	25	27
Total milk yield (TMY), L	0.317 ±0.041	0.409 ±0.043	0.406 ±0.034	0.383 ±0.039	0.385 ±0.048	0.382 ±0.037	0.358 ±0.036
Machine milk yield (MMY), L	0.127 ±0.032^{a}	0.206 ±0.034^{ab}	0.234 ±0.027^{b}	0.193 ±0.031	0.166 ±0.038	0.191 ±0.029	0.206 ±0.028
Milking time, s	52 ±4^{ab}	61 ±4^{a}	43 ±3^{b}	49 ±3	49 ±5	56 ±3	49 ±3
Milk flow latency, s	23 ±3	21 ±3	24 ±2	21 ±2	24 ±3	20 ±2	26 ±2
Milk yield in 30 s, L	0.045 ±0.024^{a}	0.060 ±0.02^{ab}	0.125 ±0.017^{b}	0.074 ±0.019	0.054 ±0.024	0.081 ±0.019	0.098 ±0.018
Milk yield in 60 s, L	0.094 ±0.030^{a}	0.151 ±0.03^{ab}	0.218 ±0.025^{b}	0.159 ±0.029	0.139 ±0.036	0.141 ±0.027	0.179 ±0.027
Machine stripping/TMY, %	54.72 ±3.63^{a}	48.82 ±3.78^{ab}	44.87 ±3.03^{b}	47.38 ±3.44^{ab}	59.67 ±4.31^{a}	44.64 ±3.30^{b}	46.19 ±3.21^{ab}
log SCC	6.367 ±0.144	6.482 ±0.166	6.462 ±0.133	6.182 ±0.138^{a}	6.211 ±0.170^{ab}	6.695 ±0.137^{b}	6.661 ±0.146^{b}

Note: ^{a,b} The means in the same line without same letter were significantly different at $p \leq 0.05$.

Table 5 Milk composition according to Somatic cell count (SCC) and milk flow type.

	SCC Group			Milk flow type			
Parameters	**≤300,000**	**300,000 – 1000,000**	**≥1000,000**	**B**	**N**	**PL**	**PLN**
N	57	15	29	37	27	23	13
Fat, %	6.26 ±0.15	5.82 ±0.27	6.26 ±0.20	6.36 ±0.17	6.36 ±0.17	5.79 ±0.21	5.87 ±0.26
Lactose, %	4.91 ±0.03	4.90 ±0.06	4.82 ±0.04	4.87 ±0.04	4.86 ±0.03	4.86 ±0.04	4.85 ±0.05
Solids, %	17.10 ±0.18	16.78 ±0.32	16.82 ±0.23	17.07 ±0.21	17.22 ±0.20	16.48 ±0.25	16.58 ±0.31
Solids not fat, %	11.05 ±0.06^{a}	11.15 ±0.11^{a}	10.75 ±0.08^{b}	10.92 ±0.07	11.12 ±0.07	10.87 ±0.08	10.89 ±0.11
log SCC	-	-	-	6.297 ±0.111	6.666 ±0.125	6.346 ±0.148	6.827 ±0.186

Note: ^{a,b} The means in the same line without same letter were significantly different at $p \leq 0.05$.

Table 6 Milk composition according to breed and milk flow type.

	Breed			Parity			
Parameters	**TS x LC**	**LC**	**IV x LC**	**3**	**4**	**5**	**≥6**
N	37	29	35	29	20	25	27
Fat, %	6.32 ±0.22	5.96 ±0.23	6.05 ±0.19	5.82 ±0.21	6.29 ±0.27	5.94 ±0.20	6.39 ±0.20
Lactose, %	4.8 ±0.05^{ab}	4.97 ±0.05^{a}	4.78 ±0.04^{b}	4.91 ±0.04	4.85 ±0.05	4.89 ±0.042	4.85 ±0.05
Solids, %	17.22 ±0.26	16.74 ±0.28	16.742 ±0.22	16.48 ±0.25^{a}	16.95 ±0.31^{ab}	16.85 ±0.24^{ab}	17.32 ±0.23^{b}
Solids not fat %	11.10 ±0.09^{a}	10.99 ±0.08^{ab}	10.87 ±0.075^{b}	10.85 ±0.09	10.87 ±0.11	11.13 ±0.08	11.10 ±0.08
log SCC	6.367 ±0.144	6.482 ±0.166	6.462 ±0.133	6.182 ±0.138^{a}	6.211 ±0.170^{ab}	6.695 ±0.137^{b}	6.661 ±0.146^{b}

Note: ^{a,b} The means in the same line without same letter were significantly different at $p \leq 0.05$.

Rovai et al. (1999) found out that the height of cisterns correlated with teat angle and distance between teats. Thus, udders with higher cisterns have deeper and show bigger teat angles. Consequently, the udder emptying can be negatively affected during machine milk, and a higher stripping fraction is observed in ewes with this udder morphology. Moreover, they found out that parity had a significant effect on cistern height **(Rovai et al., 1999)**. In SCC Group 300,000, only 14% of ewes were observed on sixth or higher lactation, but 73% in group 300,000 – 1000,000.

Table 4 shows parameters of milkability and Table 5 parameters of milk composition according to milk flow type, breed, and parity. The milk flow type had a significant effect on all tested parameters of milkability, but non on milk composition parameters. The total milk yield was lower in ewes with 1P and PL II milk flow type than in ewes with 2P and PL type in this study (Table 4). The analysis of the milk flow curves shows that the milk ejection reflex does not occur every time during milking in ewes **(Bruckmaier et al., 1997; Dzidic, Kaps and Bruckmaier, 2004; Mačuhová et al., 2008; Mačuhová et al., 2012; Tančin et al., 2011)**. 1P flow type is supposed to represent milk flow without alveolar milk ejection when only cisternal milk fraction is removed in response to machine milking **(Marnet, Negrao and Labusière, 1998; Mačuhová et al., 2008)**. This support also significantly shorter milking time ewes with 1P type of milk flow than in ewes with 2P, PL, and PL II (29 vs. 67, 54, and 66 s; resp.) as observed also in previous studies of **Mačuhová et al. (2008)** and **Mačuhová et al. (2012)** and **Tančin et al. (2011)**. 2P and in most cases also PL represent the milk flow types with milk ejection **(Mačuhová et al., 2012)**. Even the second peak is not observed in PL type of milk flow, it is supposed that milk ejection occurs in ewes with this milk flow **(Marnet, Negrao and Labussière, 1998; Rovai et al., 2002; Tančin et al., 2011)**. According to **Marnet, Negrao and Labussière (1998)**, the occurrence of the PL type of milk flow rises in consequence of the genetic selection for higher milk production or decreased

average milk flow rate. Thus, this type of milk flow can be observed when the second peak (i.e. the removal of an alveolar fraction) is masked because the cistern fraction has not yet been completely removed from the udder at the time of milk ejection **(Marnet, Negrao and Labussière, 1998)**. In the study of **Bruckmaier et al. (1997)**, high machine stripping yield was observed in ewes with 1P milk flow is possibly caused by a late response to milking and oxytocin release after the end of milking. This does not seem to be the case in this study. Machine stripping yield from total milk yield (%) was highest in PL II, whereas it did not differ among other milk flow types. However, whereas the data of this study support that no oxytocin was released during machine milking or stripping in ewes with 1P milk flow type, it is possible that oxytocin was released during milking or machine stripping in ewes with PL II milk flow type. Ewes with PL II had the lowest milk yield in 30 s (significantly) 60 s (in tendency) (Table 3). Low milk yield in 30 and 60 s could be caused by some health problems or deformity of the teat canal **(Mačuhová et al., 2008)**.

The breed influenced significantly machine milk yield, milking time, milk yield in 30 and 60 s, and machine stripping yield from total milk yield (%), and also lactose and solids not fat (Table 4 and Table 6). The machine milk yield and milk yield in 30 and 60 s were significantly higher in crossbreds IV x LC than in TS x LC (Table 4). This does not correspond to results in previous studies **(Mačuhová et al., 2008; Mačuhová et al., 2017)** where no differences were found in these parameters and also machine stripping yield from total milk yield. However, whereas the total milk yield did not differ between crossbreds in this study, machine stripping from total milk yield was higher in TS x LC than IV x LC. The reason for it could be possible worse udder morphology for milking in TS x LC ewes in comparison to IV x LC ewes of the same crossbreds observed previously.

The parity (Table 4, Table 5 and Table 6) had a significant effect only on machine stripping yield from total milk yield (%), log SCC, and solids (%). The ewes on the fourth parity have significantly higher machine stripping from total milk yield than ewes on lower or higher parities (60% vs. 47, 45, 46%). This could signalize that udder worsened with increasing parity (fourth vs. third parity). However, on higher parities (≥5) only "better" ewes (healthy, with good udder morphology, and adequate milk production) stayed in the flock.

CONCLUSION

Surprisingly, SCC Group did not affect evaluated parameters of milkability except for machine stripping yield from total milk yield (%). Milk flow type and the breed had mainly effect on the parameter of milkability. Parity had a significant effect on machine stripping yield from total milk yield (%), log SCC, and solids (%). Concerning the composition of milk, fat (%) was influenced by none of the tested parameters, lactose (%) and solids (%) only by one of the tested parameters, and only solids not fat was influenced by three (SCC group, breed, and parity) of four tested factors.

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ANALYSIS OF PORK ADULTERATION IN THE CORNED PRODUCTS USING FTIR ASSOCIATED WITH CHEMOMETRICS ANALYSIS

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ABSTRACT

Meat-based foods such as beef corned became one of the targets of counterfeiting with pork because relatively cheaper. This becomes a serious problem for Muslims, especially in Indonesia. One method that can be used to detect fat was Fourier transform infrared (FTIR) spectrophotometry. The purpose of this study was to quantitatively analyze and a group of corned beef and corned pork using FTIR spectrophotometry combined with chemometrics. Reference samples corned pork-beef made of 7 various concentration (0%, 25%, 35%, 50%, 65%, 75%, 100%) and 6 product samples purchased in the Umbulharjo, Yogyakarta. Extraction was carried out by the soxhlet apparatus using n-hexane technical solvent for 4 – 5 hours at 69 – 70 °C. Fat analyzed using FTIR spectrophotometry for generating infrared spectral data then processed with Partial least square (PLS) chemometrics for quantitative analysis and Principal component analysis (PCA) for grouping. Results of quantitative analysis chemometrics PLS, selected areas fingerprints for analysis corned pork-beef was 1180 – 730 cm^{-1} with R^2 0.9833; RMSEC 2.06%; RMSEP 1.65% and RMSECV 2.22%. The results of PCA showed groupings in different quadrants between corned pork 100% and corned beef 100%. Results showed that FTIR spectrophotometry combined with chemometrics can be used for quantitative analysis and grouping of pork corned and beef corned on the market but it can not identify pork in corned after choking process.

Keywords: corned; beef fat; FTIR; lard; PCA; PLS

INTRODUCTION

Muslims must pay attention to eat food due to it will be part of his body. Therefore, halal food is an obligation for Muslims. Halal food is defined as zero non-halal components including pork. The pork adulteration in food is used because it is cheaper than beef. This is the economic aspect reason, pork can reduce production costs **(Rohman and Che Man, 2010; Guntarti et al., 2017)**. However, we do not only consider the economic aspects. The religious aspect is an important judgment because Muslims regard pork components in processed food products is a serious problem. Islam forbade its followers to consume food products that contain pork **(Regenstein, Chaudry, and Regenstein, 2003)**.

One of the products that allow pork adulteration is beef corned. Therefore, many researchers tried to develop a halal analytical method. The triglyceride (TGA) analysis by HPLC has detection limitations because the hydrolyzed TGA can be detected by HPLC and will interfere with halal authentication **(Rohman et al., 2012[a]; Ahda, Guntari, and Kusbandari, 2016)**. FTIR spectrophotometric has been chosen because it can be combined with chemometrics to detect lard in a mixture of chicken, mutton, and veal **(Nurrulhidayah et al., 2013)**, also to detect lard in CPO and meatball product **(Ahda and Safitri, 2016; Ahda et al., 2020)** and the fat rat in beef meatballs **(Rahmawati et al., 2016)**.

The advantages of FTIR spectroscopy is an efficient analysis for detecting components in a mixture containing animal fat **(Che Man and Rohman, 2011)**. Hence, the presence of pork in processed corned beef products needs an analytical method that is accurate and precise. The use of FTIR as a halal analytical method of corned has not been reported/published. Therefore, this study was performed to determine and distinguish infrared spectra combined with chemometrics for the analysis of lard in the corned beef.

Scientific hypothesis

Lard adulteration in corned beef products can cause different vibration of the FTIR spectrum because lard contains a different TGA composition compared to beef

MATERIAL AND METHODOLOGY

Materials

Samples of pork and beef purchased at a traditional market in Yogyakarta. Spices and other additives for the manufacture of reference samples purchased in supermarkets in the District of Umbulharjo, Yogyakarta. Materials n-hexane (p.a) (Merck), n-hexane (technical) (Merck), acetone (Merck) and anhydrous Na_2SO_4.

Standard Corned Products

The corned beef was made by mixing meat, sugar, pepper, and flour. The meat was steamed for 40 minutes then packed in cans. Standard corned samples were prepared with corned beef containing 7 concentrated levels of pork in beef **(Table 1)**.

Table 1 Reference composition of Corned from beef and pork mixtures.

The concentration of corned pork (%)	Pork (grams)	Beef (grams)	Seasoning and Other Ingredients (grams)	
			Salt	Flour, Sugar, Pepper
100	90.0	0	3.0	7,0
75	67.5	22.5	3.0	7,0
65	58.5	31.5	3.0	7,0
50	45.0	45.0	3.0	7,0
35	31.5	58.5	3.0	7,0
25	22.5	67.5	3.0	7,0
0	0	90.0	3.0	7,0

Fat Extraction of Corned Products

Fat extraction was done with Soxhlet. The solvent used was n-hexane as solvent extraction. A total of 100 grams of corned beef was extracted **(Guntarti et al., 2015)**.

Fat Analysis by FTIR Spectrophotometry Method

Infrared spectra of reference samples and product samples were read by the FTIR spectrophotometer (ABB MB3000, Canada)

Data Analysis and Statistical analysis

Analysis of lard (extracted from corned beef) was carried out by spectrophotometry FTIR and processed by PLS multivariate and PCA analysis. The spectra region that showed the difference spectra of lard with other components were selected to create a model of PLS and PCA **(Miller and Miller, 2010)**.

The accuracy of the calibration models was indicated by the RMSEC and R^2 value obtained from the Horizon MB software (Philadelphia, USA). While the validation models produce RMSEP, RMSECV, and R^2 are calculated following equation below:

$$\text{RMSECV} = \sqrt{\sum_{a=1}^{n} \left(\hat{\text{Y}}\text{b} - \text{Ya}\right)^2} \Big/ \text{N} \qquad (1)$$

$$\text{RMSEP} = \sqrt{\sum_{a=1}^{n} \left(\hat{\text{Y}}\text{a} - \text{Ya}\right)^2} \Big/ \text{N} \qquad (2)$$

Where Ŷa is the actual value, Ya is the predictive value, N is the sample number, and The Ŷb is the calculated value for Ya (predictive value) based on the calibration equation with sample a **(Naes, et al., 2004)**.

RESULTS AND DISCUSSION

Design Model for Quantitative Analysis of pork in Corned

Based on the scanning, the infrared spectra showed that both pork and beef corned products contain different vibration of functional groups. The vibration C = O as the ester group was shown at wavenumber 1747 cm^{-1}. These peaks arise because the fat structure is a triglyceride consisting of the carbonyl ester group. Besides, the stretching vibration C – O group was identified at wave number 1238 cm^{-1} for beef, while pork has stretching vibration C – O group at 1234 cm^{-1}. The indication ester vibration was also illustrated at wavenumbers 1157 cm^{-1} and 1099 cm^{-1} **(Jaswir et al., 2003)**.

The vibration of the alkenes group (C = C) was illustrated at wavenumbers 3008 cm^{-1} and 1654 cm^{-1}. The different spectra produced between both pork and beef corned products showed that they have a different composition **(Figure 1)**. It can be said pork contains more unsaturated fatty acids level compared with beef. **Belitz, Grosch, and Schieberle (2009)** reported that pork contains high unsaturated fatty acids (double bonds) including oleic acid (43%) and linoleic acid (9%), while beef contains less unsaturated fatty acid than pork. Detailed vibration of all functional groups is given in **Table 2**.

However, quantitative analysis of lard in the corned products showed that the optimum difference of both pork and beef corned products was obtained at the wavenumber range of 1180 – 730 cm^{-1}. For meat discriminant, pork, beef, and mutton can be seen at 2925 cm^{-1}, 2855 cm^{-1}, and 1745 cm^{-1} with strong peaks and weak peaks at 750 cm^{-1} and 1800 cm^{-1} as fingerprint regions **(Yang et al., 2017)**. Besides, lard detection in cake formulation can be performed by FTIR at 1117 – 1097 and 990 – 950 cm^{-1} **(Syahariza et al., 2005)** and lard identification in steamed sausages and grilled sausages at wavenumbers of 1000 – 791 cm^{-1} and 1070 – 796 cm^{-1}, respectively **(Guntarti, Ahda, and Sunengsih, 2019)**. Even, lard adulteration in palm oils can be detected at 3006 and 1117 cm^{-1} **(Sim, Chai and Kimura, 2018)** or 3006 cm^{-1}, and 1120 – 1095 cm^{-1} **(Che Man, et al., 2013)** or 1481.22 – 999.05 cm^{-1} and 1793.67 – 1650.95 cm^{-1} **(Ahda and Safitri, 2016)**. Therefore, each sample type will change a marker region for lard detection. Based on the wavenumber range of 1180 – 730 cm^{-1}, the result of regression equation is y = 0.95x + 1.32, with R^2 value of 0.983 and RMSEC value of 2.055. The calibration equation is also validated using external validation and cross-validation. The results of cross-validation curve y = 0.9721x - 0.5334 with R^2 values 0.9995 and RMSECV value 2.22%. Besides, external validation has R^2 values of 0.9984, RMSEP of 1.65%, with the equation y = 0.9940x - 0.7061. the higher R^2 value and lower error (RMSEC and RMSECV) are the good indication of the obtained equation **(Rohman et al., 2012[b]; Rohman, Setyaningrum, and Riyanto, 2014; Ahda et al., 2020)**.

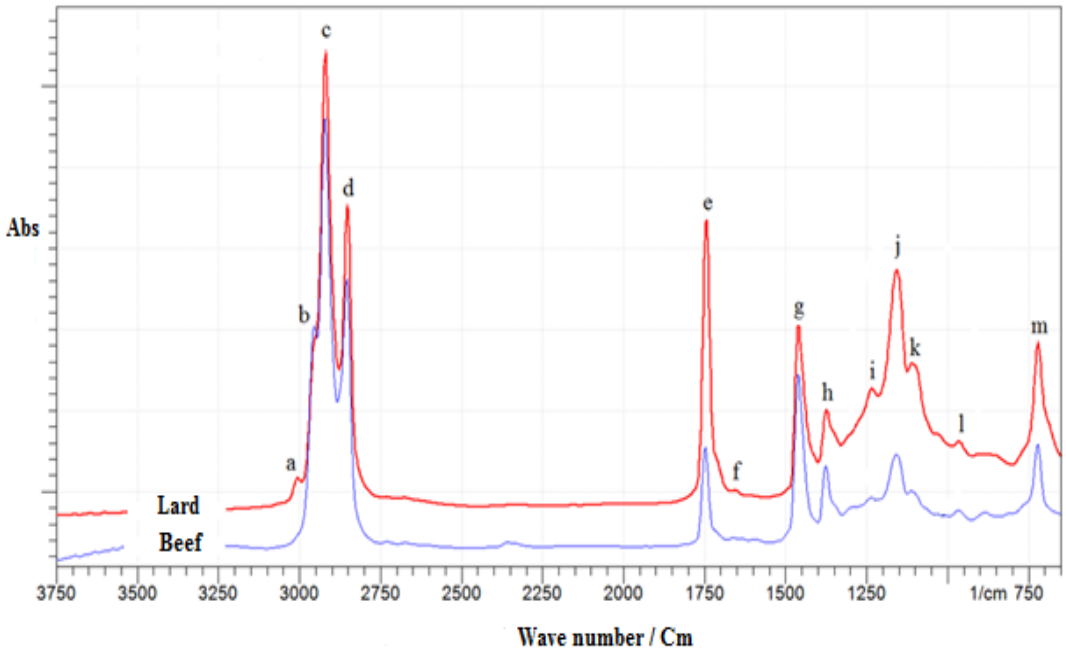

Figure 1 FTIR spectra of standard corneds from prok and beef at wave numbers 4000 – 400 cm^{-1}.

Table 2 Differences of function groups of standard corned from beef and pork.

Ribbon	Wavenumbers (cm^{-1})			Function Group	Vibration Model	Intensity
	Beef	Pork	Reference			
A	-	3008	3000	C=C-H (*cis*-)	Stretch	Weak
B	2954	2954	2960	C-H (CH_3)	Asymmetric Stretch	Medium
C	2920	2920	2930	C-H (CH_2)	Asymmetric Stretch	Strong
D	2854	2850	2850	C-H (CH_2)	Symmetrical Stretch	Strong
E	1747	1747	1750	C=O (esters)	Stretch	Strong
F	-	1654	1650	C=C (*cis*-)	Stretch	Weak
G	1461	1461	1470	C-H (CH_2, CH_3)	Cutout-Bend	Medium
H	1377	1377	1380	C-H (CH_3)	Symmetrical Stretch	Medium
I	1238	1234	1240	C-O (in esters)	Stretch	Medium
J	1157	1157	1160	C-O (in esters)	Stretch	Medium
K	1099	1099	1100	C-O (in esters)	Stretch	Medium
L	964	964	1000	C=C-H (*trans*-)	Out the Field-Bend	Medium
M	721	721	720	$-(CH_2)_n-$	Wobble-Bend	Medium

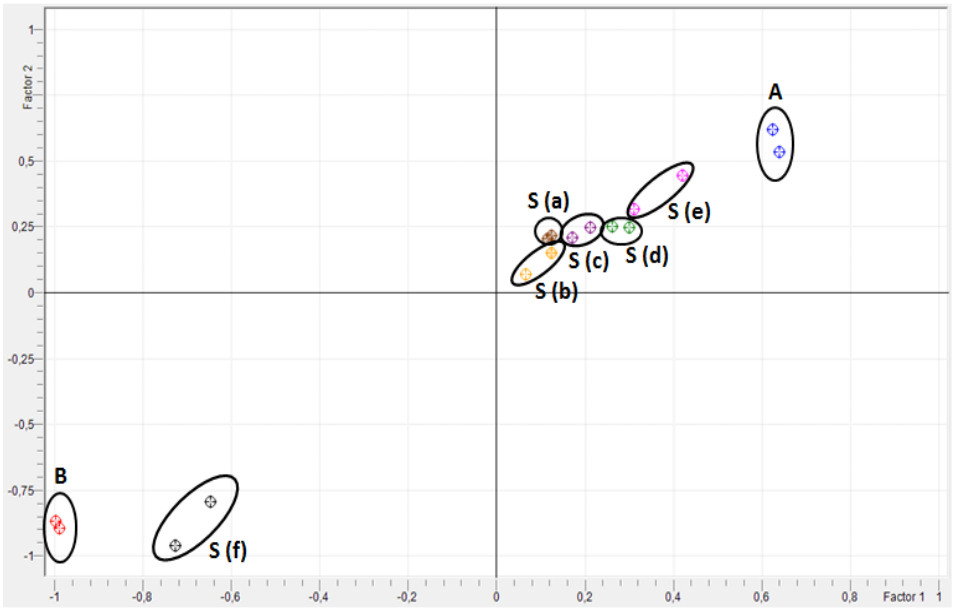

Figure 2 PCA *Score Plot* from Cornet Product Samples in the Market. Note: (A) Corned beef 100%; (B) Corned pork 100%; (S (a, b, c) Coocked commercial corned products; (S (d, e, f)) Raw commercial corned products.

Based on external validation and cross-validation were indicated that the calibration model at wave number 1180 – 730 cm^{-1} was able to give accurate results for the quantitative analysis of pork in the corned mixtures.

Analysis of Pork Adulteration in Commercial Corned Products

Analysis of pork in a food product can be performed and grouped by PCA analysis. It is an analytical technique to reduce the data when it found a correlation between the data **(Garcia, 2012)**. The PCA is unsupervised pattern recognition techniques widely used for the classification of different samples **(Nunes, 2014; Rahmania, Sudjadi and Rohman, 2015)**. PCA analysis will reduce the number of independent variables in the data to produce the new variables that are called the principal component or major components **(Che Man, Syahariza, and Rohman, 2010)**. Hence, the wavenumber regions for PCA were also optimized. Finally, the same wavenumbers used for quantitative analysis were chosen for PCA modeling due to its capability to provide good separation among the evaluated samples **(Rahayu et al., 2018; Gamperline, 2006)**.

In this study, pork analysis in the corned product is performed in optimum condition at wavenumbers of 1180-730 cm^{-1}. The discriminant analysis showed that 100% pork corned and 100% beef corned can be separated and distinguished **(Lumakso et al., 2015)**, it illustrated that both corned products have a different composition **(Figure 1)**. Therefore, analysis of pork adulteration in the commercial corned products can be performed in similar conditions **(Van der Spiegel et al., 2012)**.

In this research, we identify 3 commercial corned products and also observe the cooking effect in disrupting halal analysis. The result showed that 3 commercial corned products are not at all produced from beef. Sample (f) are grouped as pork corned, hence we can estimate that it is made from pork **(Figure 2)**. However, all samples are grouped in the beef corned product after the cooking process. It showed that the cooking process can affect the chemical properties of pork. The unsaturated fatty acids of pork may degrade during the heating process because pork corned is not separated from beef corned **(Bhaskar et al., 2012)**. Therefore, halal authentication using FTIR combined with chemometrics has a problem if the product is carried out by different process because they are possible to degrade during the process **(El-Gindy, Emara, and Mostafa, 2006)**.

CONCLUSION

FTIR spectrophotometry combined with chemometrics at wavenumbers of 1180 – 730 cm^{-1} resulted in a good correlation between the predicted value and actual value with R^2 of 0.9833, RMSEC of 2.06%, RMSEP of 1.65%, and RMSECV of 2.22%. Hence, it can be used for quantitative analysis of lard in the corned product. At wavenumbers 1180 – 730 cm^{-1}, halal authentication can be performed clearly and one of the commercial corned products was identified pork in its product. However, this method can not identify pork corned after it is cooked. Therefore, the cooking process will affect chemical compositions in commercial corned through the degradation process of unsaturated fatty acids.

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ENGINEERING MANAGEMENT OF STARTER CULTURES IN STUDY OF TEMPERATURE OF FERMENTATION OF SOUR-MILK DRINK WITH APIPRODUCTS

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ABSTRACT
The article considers the solution of problematic issues of engineering management of poly fermentation in the study of fermentation temperature of sour-milk drink with apiproducts. In the development of fermented dairy products, the components that are part of them, changes in their composition, and properties in the interconnection are considered as a technological system. The authors took into account that food technologies based on the use of the pure culture of one microorganism are limited by the capabilities of its fermentation system systems, the ultimate goal may not be achieved even by changing the conditions and parameters of cultivation. To successfully carry out fermentation processes in the technological system, a combination of cultures, associations of microorganisms with a wide range of fermentation products in contrast to one culture is promising to use. All experimental samples on a set of indicators prevailed control ones. The leader was a sample fermented with yeast with an equal ratio of cultures at a temperature of 38 – 40 °C. The authors found that the set of indicators of finished products for the production of sour-milk drinks with a complex of apiproducts, it is necessary to choose a three-strain poly fermentation product with a congruent ratio of cultures and set optimal fermentation regimes 39 ±1°C for 5.0 ±0.3 hours.

Keywords: apiproduct; sour-milk drink; fermentation temperature; technological systems

INTRODUCTION
It is known that in the development of fermented dairy products, the components that are part of raw milk, changes in their composition, and properties in the connection are considered as a technological system **(Parmjit, 2011; Palamarchuk et al., 2019)**. Its elements are independent and conditionally indivisible units that interact with each other and with the external environment **(Balduzzi and Tononi, 2008; Kozelová et al., 2011)**. The set of elements and their connections form the structure of the system, and its spatio-temporal fragments – functional subsystems **(Mda et al., 2019; Kim et al., 2015)**. The structure of the technological system for the manufacture of a sour-milk drink from apiproduct **(Grujić et al., 2011)** is presented in **Table 1**. The elements of the subsystems can function together and sequentially, in accordance with the tasks **(Giachetti, 2006; Rogovskii et al., 2019b)**. The main element of subsystem A is natural cow's milk, which provides the nutritional and biological value of the developed product **(Górska-Warsewicz et al., 2019)**. The nutritional value of milk is determined by its chemical composition, which is well studied and available in the literature **(Barłowska et al., 2011)**, is relatively stable and does not require additional research **(Rogovskii et al., 2019a)**. Milk is a structural unit of the technological system "raw milk-apiproduct-starter cultures", which is the environment of bioagents of fermentation – representatives of subsystem B **(Vieites et al., 2008)**. The result of this process is the biotransformation of components of subsystems A and B, which ensures the quality and properties of the future product **(Nikolic, 2018)**. The elements of subsystem B are lyophilized lactic acid microorganisms with known characteristics **(Table 2)** and therefore it did not require in-depth study **(Azat et al., 2016)**. In contrast to the components of subsystem B (apiproducts), which required careful examination, their characteristics may change over time **(Bishop et al., 2012)** and botanical origin **(Bober et al., 2020)**.

Scientific hypothesis
The scientific hypothesis assumes the existence of functional dependence in the fermentation process of three types of associations of lactic acid bacteria in the technological system with a complex of apiproducts and without them. The fermented milk process should be more active in the experimental media, as the hydrogen index should be lower than in the control ones.

Table 1 The structure of the technological system of sour-milk drink with apiproducts.

Subsystem	Elements of subsystems	Subsystem components
A	The components, that regulate the nutritional, biological and energy value of sour milk drink, are the living environment of biological objects	Raw milk
B	Components (growth factors) that regulate the activity and viability of biological objects	Apiproducts
C	Bioobjects that regulate fermentation	Starter cultures

Table 2 Characteristics of lactic acid bacteria.

Lactic acid bacteria	Temperature optimum, ºC	Interfacial acidity, ºT	Coagulation time, hour	Temperature range, ºC
Lactococcus lactis	25 – 30	125*	4 – 6	10 – 40
S. thermophilus	40 – 45	110 – 115	3.5 – 4	20 – 50
L. acidophilus	37 – 38	260 – 280	5 – 8	5 – 53
L. bulgaricus	40 – 45	200 – 350	4 – 6	20 – 50
B. longum	36 – 40	120 – 130	10 – 12	20 – 50

Note: * – achieved in 5 – 7 days.

MATERIAL AND METHODOLOGY

Given that food technology, based on the use of the pure culture of one microorganism, is limited by the possibilities of its enzyme systems, the ultimate goal may not be achieved even by changing the conditions and parameters of cultivation for ISO 27205:2010 **(ISO 27205:2010)**. Biological systems with a wider internal variety of signs, properties, and qualities have higher viability. For the success of fermentation processes in the technological system, it is promising to use a combination of cultures, associations of microorganisms with a wide range of enzymes in contrast to one culture.

The next series of experiments were conducted for a comparative study of technological processes in normalized milk (control technological system) and the milk environment with the complex of apiproduct (experimental technological system) with the participation of poly ferments of different bacterial composition.

First of all, two variants of associations were studied: the first consisted of three cultures – *S. thermophilus*, *L. acidophilus*, *L. bulgaricus*. The second, in addition to these, additionally contained a fourth species of microorganisms – *Lactococcus lactis* **(Table 3)**.

Table 3 Scheme of the presence of cultures in associations.

Component of the technological system	Samples			
	K1	K2	D1	D2
Prepared cow's milk	+	+	+	+
Complex of apiproducts	–	–	+	+
S. thermophilus	+	+	+	+
L. acidophilum	+	+	+	+
L. bulgaricus	+	+	+	+
Lactococcus lactis	+	–	+	–

Statistic analysis

Statistical analysis of the results of experimental studies was performed in five replicates using standard methods of research of organoleptic, physical, physicochemical, microbiological, and other indicators. The obtained results of experimental research are processed using modern analytical integrated systems Microsoft Excel 2016 and Statistica 13.3. Adequacy of decision-making was carried out according to the criteria of Fisher, Cochran, and Student.

RESULTS AND DISCUSSION

Four samples took part in the experiment: two control samples – K1 and K2, which did not contain apiproduct and two experimental samples – D1 and D2, obtained by fermentation of the technological system "raw milk-apiproduct-starter cultures". Similar studies were conducted by the authors of the following scientific papers **(Banik et al., 2003; Abdul Manan and Webb**, **2017; Montemurro et al., 2019)**. The ratio of cultures in fermentation products was congruent within each, incubation was performed at a temperature of 39 – 40 °C. In scientific works **(Ospina, Merrill and Benson, 2018; Smith et al., 2018)** incubation was performed at a temperature of 28 – 34 °C. Titrated and active acidity was determined within 6 to 12 hours every 60 minutes. In scientific works (**Shanina et al., 2019; Wijnen et al., 2020**) titrated and active acidity were determined for seven hours with an interval of 30 to 120 minutes. Samples D1 and D2 were compared with control ones **(Figure 1)**.

Studies of acid-forming activity in control and experimental environments have shown that apiproduct has a positive effect on the fermentation process, accelerating it **(Todorov et al., 2017)**. In particular, cultures grown in experimental technological systems with apiproduct overcame the stage of preparation of microorganisms for reproduction (lag phase) 2 times faster. This was more noticeable in the example of sample K2 **(Furtado et al., 2014)**. This may be due to the lower acid-forming capacity

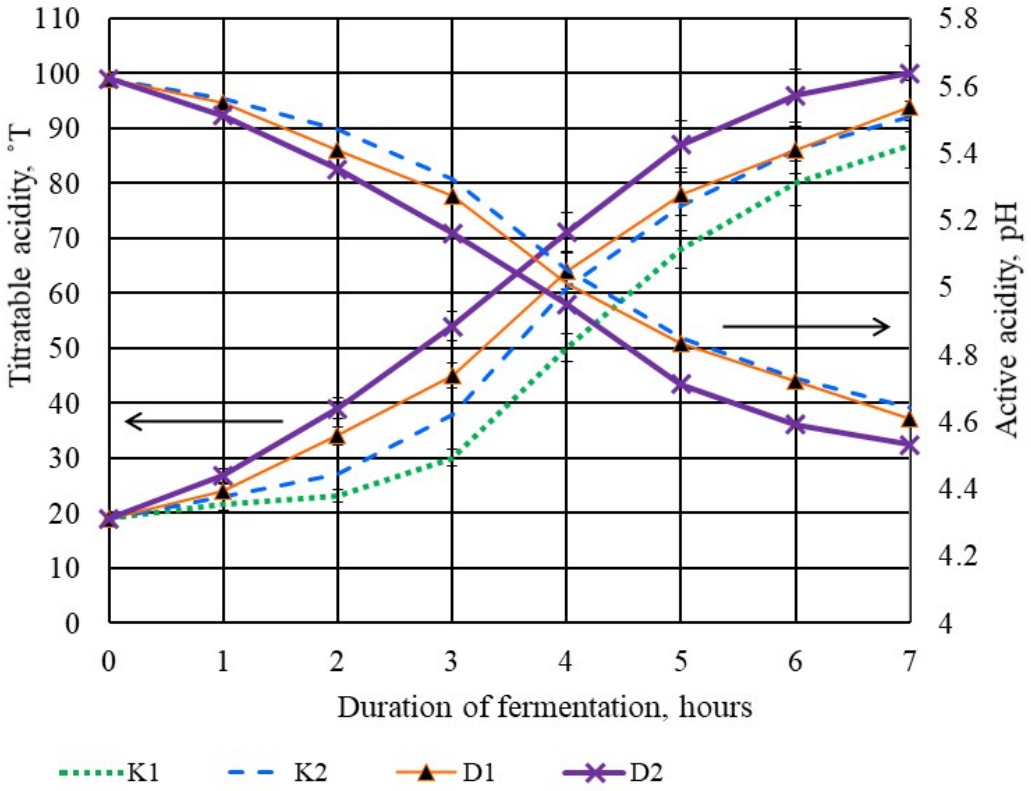

Figure 1 Dynamics of acidity of technological systems.

Table 4 Organoleptic parameters of sour-milk clots under the action of various associations of microorganisms.

Sample	Organoleptic parameters	
	consistency and color	taste and smell
K1	Moderately dense, slightly brittle and viscous	Pure, sour-milk, fresh taste
K2	Viscous, dense, homogeneous	Sour milk, moderately sour
K3	Homogeneous, viscous	Taste and smell are satisfactory
K4	The texture is viscous, slightly heterogeneous	Taste and smell are satisfactory
D1	The yellow clot is homogeneous, moderately dense	Sweet, not sour enough with a pleasant taste and smell of honey and pollen
D2	The clot of pleasant yellow color, homogeneous, dense, viscous	Harmonious sweet and sour, with a pleasant taste and smell of apiproducts
D3	Viscous sour milk gel with a yellow tint	Taste and smell are satisfactory
D4	Texture and color are satisfactory	Taste and smell are satisfactory

of *Lactococcus lactis* compared to *L. acidophilus* and *L. bulgaricus*. The reduction of the lag phase is a positive phenomenon, as it will reduce the likelihood of the development of unwanted microflora in the initial stages of fermentation of the technological system **(Sahu and Panda, 2018)**, when the acidity has not risen to a safe level yet **(Adebo et al., 2017)**.

The phase of smooth growth of biomass (log-phase) was also more productive in the technological system with the apiproduct complex for both starter cultures complex, but the association, which contained *Lactococcus lactis*, lagged behind the speed and efficiency of fermentation at all stages of microflora development **(Singh et al., 2015)**. So, the starter cultures complex, which includes *S. thermophilus, L. acidophilus, L. bulgaricus*, proved to be better in both control and experimental environments **(Glušac et al., 2015)**. To justify the choice of one of the two options for combinations of poly fermentation products **(Dafeo and Daugulis, 2013)**, an additional organoleptic evaluation was performed **(Table 4)** and the ability of the sour-milk drink to emit the moisture (syneresis) was compared.

It was found that the use of different ferments in the presence of apiproduct in milk provides products with different flavors **(Widyastuti et al., 2014)**. Thus, sour-milk drinks obtained by fermentation of the technological system "raw milk-apiproduct-starter cultures" had an appetizing appearance, pleasant color, pure sour-milk-honey smell, but the taste was not harmonious enough due to insufficient acidity. Sample D2, obtained by using three cultures (*S. thermophilus, L. acidophilus, L. bulgaricus*) had an appetizing appearance, pleasant sour-milk-honey aroma, and harmonious taste **(Spence et al., 2017)**.

The synergistic properties of sour-milk gels obtained by fermentation of the technological system "raw milk-apiproduct-starter cultures" indicate in favor of a three-strain combination of microorganisms, as sour-milk drinks, obtained by its use, better retain moisture in their structure **(Figure 2)**.

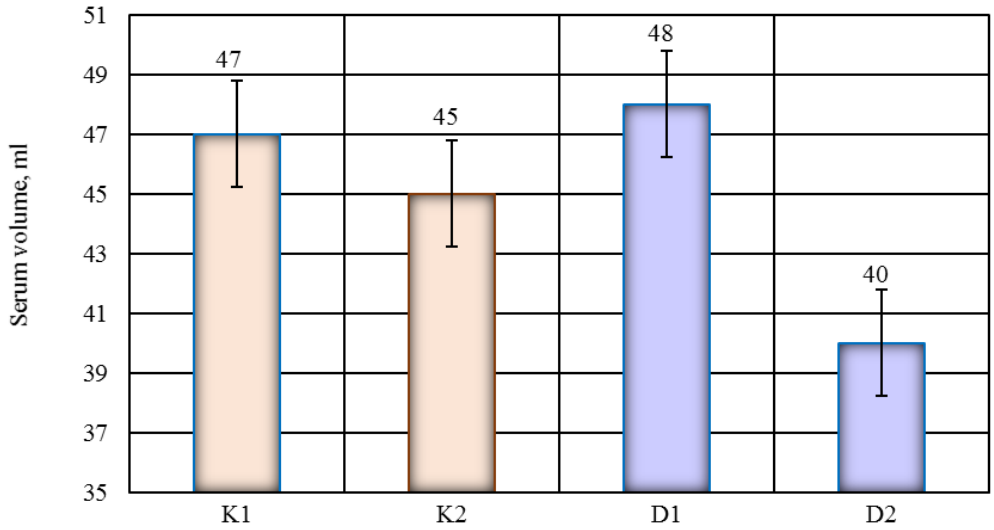

Figure 2 Synergetic properties of fermented technological systems.

Table 5 The structure of technological systems for studying the properties of associations of microorganisms.

Component of the technological system	Samples							
	control				experimental			
	K1	K2	K3	K4	D1	D2	D3	D4
Prepared cow's milk	+	+	+	+	+	+	+	+
Complex of apiproducts	–	–	–	–	+	+	+	+
2/4 *S. thermophilus*, 1/4 *L. acidophilus*, 1/4 *L. bulgaricus*	+	–	–	–	+	–	–	–
1/3 *S. thermophilus*, 1/3 *L. acidophilus*, 1/3 *L. bulgaricus*	–	+	–	–	–	+	–	–
1/4 *S. thermophilus*, 1/4 *L. acidophilus*, 2/4 *L. bulgaricus*	–	–	+	–	–	–	+	–
1/4 *S. thermophilus*, 2/4 *L. acidophilus*, 1/4 *L. bulgaricus*	–	–	–	+	–	–	–	+

After 3 hours of filtration of the 100 ml sample, less serum was released in K1 samples than in D1 by 1 ml and in K2 more than in D2 by 5 ml. The difference between samples K1 and K2 was 2 ml (4 %), and between D1 and D2 – 8 ml (16.7 %). Incubation of four-strain starter cultures complex in the medium with apiproducts led to a deterioration in the ability of the gel, formed by it, to retain moisture in the structure **(Mushtruk et al., 2020)**.

This is evidenced by a 2 % increase in the compression of the dispersion medium of sample D1 compared with K1. Thus, sour-milk gels formed by the starter cultures consisting of *S. thermophilus*, *L. acidophilus*, *L. bulgaricus*, retain moisture better than those formed by *S. thermophilus*, *L. acidophilus*, *L. bulgaricus*, *Lactococcus lactis*, the difference becomes more noticeable during the fermentation of the technological system "raw milk-apiproduct-starter cultures". This may be due to the ability of the *L. acidophilus*, *L. bulgaricus*, *Lactococcus lactis* to form a sour-milk gel with a brittle structure that displaces the dispersion medium. In contrast to lactic acid mucus-forming rods (*L. acidophilus*, *L. bulgaricus*) and thermophilic streptococcus, which form clots of viscous and creamy consistency **(Sfakianakis and Tzia, 2014)**, which provides low self-expression of serum.

A comparative evaluation of the action of two starter cultures by the set of signs of leaven, in dairy environments with a complex of apiproduct and without it, suggests that in further studies we should use the first of them. Because the combination of *S. thermophilus*, *L. acidophilus*, and *L. bulgaricus* provides the best quality in organoleptic, biochemical, technological, and others. properties of the product than poly fermentation product with the composition: *S. thermophilus*, *L. acidophilus*, *L. bulgaricus*, *Lactococcus lactis*.

Substantiation of fermentation regimes and composition of poly cultural leaven **(Kennedy and Krouse, 1999)**. The biochemical activity of microorganisms should be studied under the conditions of a set of influencing factors: the composition of the nutrient medium, the temperature of cultivation, the composition of the leaven **(Beltran et al., 2008; Bober et al., 2020)**. The influence of the first factor was investigated using two variants of media: the first – normalized milk with a mass fraction of fat of 3.2 %, pasteurized at a temperature of 87 ±2 ºC, and cooled to fermentation temperature, the second – the same milk with a complex additive. The effect of the second factor was investigated by varying the cultivation temperature **(Table 2)** from 38 °C to 42 °C in increments of 2 °C. Temperatures above 42 ºC are undesirable for the apiproduct. The influence of the third factor was studied by changing the ratio of cultures in the starter cultures. As a result of the technological system of design, eight samples of sour-milk clots were obtained **(Table 5)**: control – K1-K4 and experimental – D1-D4.

As a result of designing the technological system, eight samples of sour-milk clots were obtained, which differed in the presence of apiproduct in their composition and were fermented by different. Lactic acid bacteria (LAB) associations **(Fahrurrozi et al., 2019)**. Control and experimental samples were evaluated by coagulation time

and titrated acidity **(Marchi et al., 2009; Zheplinska et al., 2020)**. The research results are presented in **Table 6.**

Analyzing the biochemical activity of the association of lactic acid cultures in control and experimental of the technological system at a cultivation temperature of 38 – 42 ºC, we can conclude that, under these conditions, environments with apiproduct complex performed better than control ones.

The results of the study of culture activity under different cultivation conditions showed that an increase in biomass was obtained in environments with apiproducts, which is higher than in the control ones by 7.4 %. In scientific works **(Barãoa et al., 2019; Abdulgader et al., 2019)** descriptive experiments where the increase in biomass obtained in environments with a variety of products ranged only at the level of 3.5 - 4.6%.

Increasing the fermentation temperature by 2 °C causes an increase in acidity by 1 °T, a similar effect on the content of Log CFU ml^{-1} is not observed.

The number of lactic acid bacteria grown at a temperature of 3 ºC was higher than the number grown at a temperature of 40 ºC by 1.0% in the control group and 2.0% in the experimental group.

A further increase in the fermentation temperature to 42 ºC caused an inverse reaction, which resulted in a decrease in the number of viable cells, both in the control and in the experiment, by an average of 3.4 %, compared with samples cultured at 40 ºC by 2 % compared to those samples that were fermented at a temperature of 38 ºC.

As the fermentation temperature increases, the acidity of the clots increases – protein coagulation is faster by almost one hour, but under such conditions, there is a tendency to reduce the synthesis of biomass.

The time of clot formation has a directly proportional effect on the duration of the production process, and acid coagulation of milk correlates with the level of organic acids in the environment.

However, with the acceleration of coagulation, as can be seen from **Table 6**, the ability of microorganisms to

Table 6 Activity of cultures under different conditions of cultivation, $n = 3$, $p \leq 0.05$

Sample	Titrated acidity, °T	Time of Gel formation, hour	Cell numbers log LAB/g
Cultivation temperature 38 °C			
K1	85 ±1	5.5 ±0.3	8.7 ±0.2
D1	90 ±3	5.0 ±0.1	9.4 ±0.1
K2	84 ±2	5.6 ±0.2	8.8 ±0.1
D2	89 ±3	5.4 ±0.2	9.3 ±0.2
K3	88 ±1	5.3 ±0.5	8.5 ±0.2
D3	92 ±1	4.8 ±0.3	9.3 ±0.1
K4	87 ±2	5.4 ±0.3	8.6 ±0.1
D4	91 ±3	4.9 ±0.1	9.3 ±0.1
Cultivation temperature 40 °C			
K1	86 ±1	5.4 ±0.1	8.8 ±0.1
D1	91 ±1	4.7 ±0.1	9.5 ±0.2
K2	85 ±1	5.5 ±0.2	8.9 ±0.3
D2	89 ±2	4.9 ±0.1	9.3 ±0.3
K3	89 ±2	5.2 ±0.1	8.7 ±0.2
D3	94 ±2	4.5 ±0.2	9.3 ±0.2
K4	88 ±3	5.3 ±0.3	8.5 ±0.3
D4	92 ±1	4.6 ±0.2	9.3 ±0.1
Cultivation temperature 42 °C			
K1	88 ±1	4.8 ±0.1	8.5 ±0.1
D1	93 ±2	4.0 ±0.1	9.3 ±0.3
K2	86 ±2	4.9 ±0.2	8.5 ±0.2
D2	90 ±1	4.2 ±0.2	9.2 ±0.1
K3	90 ±2	4.6 ±0.2	8.4 ±0.1

Table 7 Characteristics of products obtained under different fermentation regimes.

Sample	Fermentation temperature								
	38 ºC			40 ºC			42 ºC		
	score	viscosity, sec.	pH	score	viscosity, sec.	pH	score	viscosity, sec.	pH
K1	5	83 ±1	4.7 ±0.03	4.9	83 ±2	4.7 ±0.02	4.9	84 ±1	4.6 ±0.09
D1	5	84 ±1	4.6 ±0.07	5.0	84 ±2	4.6 ±0.05	4.9	84 ±3	4.6 ±0.03
K2	4.9	70 ±2	4.7 ±0.05	4.9	71 ±3	4.7 ±0.03	4.8	73 ±2	4.7 ±0.02
D2	4.9	79 ±1	4.6 ±0.08	4.9	80 ±3	4.6 ±0.07	4.8	80 ±2	4.6 ±0.07
K3	4.4	80 ±2	4.6 ±0.09	4.4	81 ±1	4.6 ±0.08	4.3	80 ±3	4.6 ±0.07
D3	4.7	83 ±1	4.6 ±0.04	4.7	83 ±3	4.6 ±0.03	4.6	83 ±1	4.6 ±0.01
K4	4.5	80 ±1	4.7 ±0.01	4.5	80 ±2	4.6 ±0.09	4.4	81 ±1	4.6 ±0.08
D4	4.8	82 ±2	4.6 ±0.05	4.7	82 ±2	4.6 ±0.04	4.6	82 ±1	4.6 ±0.01

accumulate biomass decreases, and the number of viable cells decreases, both in control and in the experiment.

For example, in the control of acidity 85 °T Log CFU ml^{-1} is 8.7, and at an acidity of 88 °T – 8.5, and the time of clot formation at the specified acidity is reduced by 13 %. Similar dynamics are observed in the experimental samples, but the loss of cell concentration for every 1 °T is twice lower. It should be noted that coagulation should not be accelerated while neglecting the probiotic properties of the sour-milk drink. Therefore, the recommended fermentation regimes are 39 ±1 ° C for 5.0 ±0.3 hours of fermentation. Analyzing the results of the model associations of microorganisms, we can conclude that the best results were achieved by those who had a congruent ratio of cultures (K1, D1), both in environments with apiproduct and without them.

Studies of organoleptic, physicochemical, and rheological properties of cultures of lactic acid organisms under different cultivation conditions are presented in **Table 7**.

The data obtained indicate that with increasing fermentation temperature, there is a tendency to accelerate the decrease in the concentration of hydrogen ions and a slight increase in viscosity. A more pronounced difference was observed in the number of points scored between control and experimental samples, fermented under different conditions **(Woo et al., 2019)**. An increase in the cultivation temperature by 2 °C causes such negative phenomena as excessively sour taste, intense serum secretion, minor defects in the consistency of the sour-milk clot (flakes, etc.) and it leads to a decrease in the overall score by an average of 0.4 points for all samples.

Thus, the results of the research of organoleptic, physicochemical, and rheological properties of LAB cultures under different cultivation conditions indicate that fermentation should be carried out at a temperature of 38 – 40 ºC.

CONCLUSION

During the study of the fermentation process of three species associations of lactic acid bacteria in technological systems with a complex of apiproducts and without them, it was noted that the sour-milk process is more active in the experimental environment because the hydrogen index was lower than in control ones.

Sensory evaluation of sour-milk drinks showed that the best taste properties had experimental samples. This is because the sweet taste of honey was harmoniously combined with sour-milk, and the floral aroma of the apiproduct successfully emphasized the characteristic smell of fermented dairy products. The highest score among the experimental samples scored D1 and D2, but the relative viscosity of the sample D1 was higher by 6 % and was 83 – 85 seconds.

All experimental samples (D1 – D4) in the set of indicators prevailed control ones (K1 – K4), but the leader was the sample D2, fermented with an equal ratio of cultures at a temperature of 38 – 40 °C.

Therefore, according to the results of the study of the set of indicators of finished products for the production of sour-milk beverages with a complex of apiproducts, a three-strain poly fermentation product with a congruent ratio of cultures was selected and optimal fermentation regimes were established: 39 ±1 ºC for 5.0 ±0.3 hours.

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FORMING THE SYSTEM OF FOOD SECURITY INDICATORS FOLLOWING THE CRITERIA OF THE SDGS-2030

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ABSTRACT
Effective management of any system is impossible without a clear definition of its elements, hierarchical levels, and desired performance indicators. Considering this problem in the context of food security management, the authors set out to create a system of food security indicators at different hierarchical levels. The purpose of the article is to deepen theoretical and methodological provisions and develop a system of indicators as a component of food security management at different levels, which should meet the criteria and dimensions of the SDGs-2030. The methodological basis of the research is the dialectical method and general scientific and special methods of scientific knowledge. The results obtained are of great practical importance in shaping national and regional food security policies (programs) on a sustainable development basis. Proceeding from the results of the research and the scientific and theoretical positions of the epistemological content of the category of the concept "food security", taking into account the criteria of food security of formation at different hierarchical levels and methodological aspects of its monitoring, it is proposed 54 indicators.

Keywords: food security; indicator; level; method; system

INTRODUCTION
The process of food security should be accompanied by organized monitoring of the nature of the changes, their quantitative and qualitative assessment to prepare the appropriate recommendations, and management decisions. The monitoring system is based on a combination of economic and social indicators with indicators that reflect the results of activities of state authorities in food security. According to the monitoring results, the executive authorities should decide on changes to the food basket for the main social and demographic groups of the population, and the executive authorities should make decisions on changes and approve sets of food products for the main social and demographic groups **(Verkhovna Rada of Ukraine, 2007a)**.

In the context of European integration, which involves openness of the internal and external markets of food, it is necessary to monitor the indicators of food security of the country at different hierarchical levels constantly, which will allow to react promptly to changes and to formulate corresponding state, regional and local policies.

Modern science does not provide a unified indicator system for food security monitoring. A large number of studies, devoted to this issue, indicates its relevance and the practical impossibility of the unification of indicators' system for all regions, subregions, and countries. It is obvious that the estimated indicators are not constant – they depend on the desirable criteria and specific features of a particular region, which ultimately forms the specific tasks of the state policy of each country to achieve food security grounding on sustainable development.

Ukraine does not have a methodological approach to the food security monitoring, including the all level, which should meet the criteria and dimensions of the SDGs-2030.

Scientists have made a considerable effort over the decades to create the best set of indicators for assessing food security and nutrition safety.

The overwhelming majority of scientific works on monitoring of food security is devoted to detailed research of specific aspects of the issue, for example:

(1) prevalence of micronutrient malnutrition **(Ramakrishnan, 2002)**;

(2) focus on three groups of risk factors: behavioral, environmental and occupational, and metabolic **(Forouzanfar et al., 2015)**;

(3) a comprehensive dietary analysis of low-income adults and examined differences in dietary intake between SNAP participants and nonparticipants **(Leung et al., 2012)**;

(4) examine the association between household food insecurity and the likelihood of perceived clinical malaria among 1- to 5-year-old children living in rural south Haiti **(Pérez-Escamilla et al., 2009)**;

(5) underlying mechanisms of under-and over-nutrition among children in rural China **(Zhang et al., 2016)**;

(6) summarizes the literature on the link between food insecurity and the following diet, weight gain, and chronic disease, especially among women **(Laraia, 2013)**.

These studies are local in terms of geography (a specific place of residence or region is being investigated) or have component problems (only several aspects are being investigated). The combination of all mentioned in these works (and many other) problems into a single system of indicators for monitoring food security is not meaningful, since most of them are relevant only for specific areas or groups of the population.

Some works are devoted to global research on the achieved level of food security. In particular, **Smith, Rabbitt and Coleman-Jensen (2017)** identify and examines the common determinants of food insecurity in 134 countries using cross-country comparable experiential of food insecurity.

This approach is expedient when it comes to assessing the level reached by countries, continents, or mainlands in comparison to the level of food security at the global level. **Pérez-Escamilla and Segall-Corrêa (2008)** give an overview of the advantages and disadvantages of five commonly used methods that can be used to assess food security. This study is intended to help researchers in choosing methods, taking into account their advantages and disadvantages, in monitoring food security.

Summarizing, we can conclude that the methodology of food security monitoring is based on the following basic components: problems, features, location, and methods. The indicators' system of food security monitoring should provide coverage of these components. That is why indicators of food security monitoring at the global level are inadequate in determining food security at the level of region or individual – they have different levels of problems and opportunities. So, we support the viewpoints of the scholars, **Frankenberger and McCaston (1998)** and **Gross et al. (2000)** in particular, who examine food security through the prism of hierarchical levels: global, national, households and individuals. This approach allows taking into account all the peculiarities of the formation of the food chain both of the locality (resource supply, specialization, and natural conditions) and personality (incomes, tastes, individual needs).

Global level

FAO provides information on 27 indicators of food security on the SDG-2030 **(FAO, 2019a)**; according to other criteria of the Agenda for the period until 2030, the system of indicators is under development. Therefore, an assessment of the state of food security at the global level and FAO and scientists make due to the indicators proposed by FAO in theprocessntation of the new Sustainable Development Goals (SDGs-2).

FAO is the custodian UN agency for 21 SDG indicators and is a contributing agency for a further 4. In this capacity, FAO is supporting countries' efforts in monitoring the 2030 Agenda **(FAO, 2019b)**.

Proposals of national scientists regarding the methodology for assessing food security at the global level are practically absent. Some of them are borrowed from individual indicators developed by FAO for use in the national system for monitoring food security.

National level

At the state level, the assessment of food security is carried out following the Methodology for determining the main indicators of food security, approved by the Cabinet of Ministers of Ukraine Resolution "Some issues of food security" No. 1379 dated December 5, 2007 **(Cabinet of Ministers of Ukraine, 2007) (Table 1)**.

Proposals of national scientists regarding the improvement of this Methodology for defining the main indicators of food security are different. However, some of them require a radical change of methodology for calculating the indicator, and chage the value of the margin, and deeper analysis (calculation of additional indicators) in interpreting the results. So, **Nud (2013)** "believes that the 60% threshold for the indicator of economic availability of products is too high".

Another normative document, which defines food security indicators, cannot be overlooked – The Law of Ukraine "On Food Security of Ukraine" **(Verkhovna Rada of Ukraine, 2007a)**. Currently, this document has the status of a project, and therefore official food security reports are based on the indicators contained in the Methodology for defining key indicators for food security.

We partially agree with several comments on food security indicators in the Conclusion on the draft Law of Ukraine "On Food Security of Ukraine" **(Verkhovna Rada of Ukraine, 2007b)**.

Such an indicator of food security, determined in clause 3 of Part 1 in article 7, as "provision of grain stockse food resources, defined as the ratio between the volumes of food grains in such resources and the volume of national consumption of bread and grain products in the calculation of grain". It is expedient, in our opinion, to supplement it with additional Corrective indicator – "grain production per person per year, tons". Such indicator is provided by the Methodical recommendations for calculating the level of economic security of Ukraine **(Ministry of Economic Development and Trade of Ukraine, 2013)**. Secondly, it reduces the impact on the calculation of the marginal indicator of the adequacy of grain supplies in public food resources of such factors as the level of solvent demand **(Verkhovna Rada of Ukraine, 2007a)**.

Grishova and Kryukova (2014) defined seven indicators and their corresponding normative values as the main indicators of food security. The authors also suggested a generalization of the system of indicators of the level of food security of the country using the integral coefficient.

The authors ranked the most significant integral indicators taking part in the formation of the integral index of food security level: the volume of grain production per capita, which forms the potential fund of population consumption; the level of economic availability of food; the share of food imports in the structure of consumption of the domestic market; the average wage as one of the main sources of household income.

In our opinion, this approach is appropriate in determining changes in the long-term dynamics or ranking of regions of Ukraine. The list of indicators is too large, and the method of their calculations is complicated.

Table 1 Methodology for determining the main indicators of food security.

Name of the indicator	Method of calculation	Limit (threshold) criterion
Daily energy value of a human diet	$EV = \Sigma m_i z_i$, where EV – energy value of the daily ration of a person; ia – type of food; mi – a mass of the i-th product consumed by one and the person; zi – energy value of the mass of the i-th product;	2500 kcal per day, 55% of the ration should be provided at the expense of products of animal origin
Ensuring the human diet by main types of products	$C = \frac{C_f}{C_r}$, where C – adequacy indicator consumption of certain products; Cf – actual consumption of a single product per person per year; Cr – rational norm of consumption of a separate product kg per person per year, agreed with the Ministry of Health	*
Availability of grain stocks in state resources	$G = \frac{H}{X}$, where G – indicator of grain security by food resources; H – availability of food grain in the state food reserve; X – average annual internal consumption of bread and grain products in terms of grain	17%, corresponding to 60 days of consumption
Economic availability of products	$E = \frac{B_x}{B_c}$, where E – an indicator of the economic availability of products; Bx – population expenditures for food per year; Bc – total expenditure of the population for the year	60%
Differentiation of the cost of food by social groups	$D = \frac{D_b}{D_m}$, where D – an indicator of the differentiation of the cost of food; Db – the value of consumed products in the 20 percent of households with the highest incomes; Dm – an indicator of the cost of consumed products in 20 percent of the lowest-income households	-
The capacity of the internal market of individual products	$V_i = F_i N$, where Vi – capacity of the domestic market of the i-th product; i – a type of the i-th product; Fi – annual average consumption of the i-th product; i N – average annual population	-
Food independence for a particular product	$P = \frac{I_i}{V_i}$, where P – a share of food imports of the i-th product; i – a type of i-th food product; Ii – imports of the i-th product; Vi – capacity of the domestic market of the i-th product	30%

Note: * Bread products – 101; meat and meat products – 80; milk and dairy products – 380; fish and fish products – 20; eggs (pcs.) – 290; vegetables and food crops – 161; fruits, berries and grapes (without wine processing) – 90; potatoes – 124; sugar – 38; vegetable oil of all kinds – 13. Source: **Some issues of food security (2007)**.

It is possible to avoid these errors, in our opinion, if when developing a system of food security assessment indicators, take into account the criteria for the functioning of the system.

Banakh (2016) defines five basic food security criteria and their respective indicators for statistical analysis: physical accessibility; economic availability; social accessibility; food independence; ecological factor. **Stezhko (2014)** forms a system of indicators of the state of food security, based on three criteria (although the author calls them the direction of evaluation, and as criteria defines different indicators): physical availability of food; economic availability of food; food for consumption.

It should be noted that both **Banakh (2016)** and **Stezhko (2014)** proposed to add the indicators to the system. These are soil fertility indices (environmental factor, **Banakh (2016)**) and overweight population (food security for consumption, **Stezhko (2014)**). The first indicator is one that characterizes the degree of sustainability of agricultural development – a criterion that domestic scientists practically do not take into account. At the same time, sustainable development of agriculture is an integral part of SDG2, along with food security. The second indicator is also ignored by academics since it is believed that food security is the lack of calories, and not over their daily norm, which also leads to overweight. At the same time, in other equal terms, such an indicator simply indicates the lack of economic access to food and the replacement of quality products with cheap ones, which in turn contain so-called "empty" calories, which do not allow to satisfy the need for the necessary minerals and vitamins but overload the body with carbohydrates, starch, and others.

Another important aspect for scientists is the question of the possibilities and limits of the use of food security assessment methodology in other countries. Of course, some indicators or methodologies, or methodological approaches can be borrowed, but their use in the national system for assessing food security should be justified and appropriate. Otherwise, in the end, we will have a large block of indicators, which will be difficult to interpret. There is another problem related to the methodology of collecting and processing statistical information, which has differences in each country. On January 10, 2003, Ukraine officially became the 52nd country (the first among the countries of the Commonwealth of Independent States), which joined the International Monetary Fund's Special Data Dissemination Standard. However, the list containing the IMF Special Data Dissemination Standard includes a limited list of indicators, most of which do not even indirectly reflect the state of food security. **Volchenko (2013)** introduced such indicator systems in the Russian Federation and Belarus as examples of foreign experience in the formation of the country's food security system. At

the same time, we should pay attention to the fact that the data of the systems of indicators of food security assessment of the countries are based on the criteria laid down in the approved Doctrine (RF) and the Concept (Belarus), which are aimed at achieving the goals that do not coincide with the goals that Ukraine sets itself.

We can accept may the experience of foreign researchers who propose establishing an optimal level of indicators for food security. So, **Bekenov (2003)**Error! Reference source not found.**, Ilyna and Kondratenko (2007)** determine the levels of optimality of food security indicators, but in the first and second cases it is only one indicator.

Russian explorer **Ogluzdin (2010)** proposes applying a five-point system of assessments of the state of food security. At the same time, it should be noted that it is not clear how to determine some of the proposed scientific indicators, because they sound, rather, as criteria-tasks than specific indicators. Secondly, not all indicators have limit levels defined, and therefore assign them to one of the five groups is not so easy. Thirdly, the list of indicators is too large and involves, in practice, the calculation of most of the indicators of economic, and not just food security. Instead, there are no specific indicators that determine the level of food security in the system.

We support the opinion of scientists who believe that the methodology for assessing food security indicators involves identifying their optimal, threshold and marginal levels, where the optimal level of food security indicators represents the range of values within which the most favorable conditions for reproductive processes in the national agro-industrial production are created; the threshold level is determined by the value of quantitative values, the violation of which causes unfavorable tendencies; marginal level – the limit violation of which causes threatening processes and trends in the system.

Research shows that the overwhelming majority of scientists define only the threshold levels of indicators. Some researchers' suggestions relate only to individual indicators. Thus, according to the classification proposed by **Kochetkov and Markov (2002)**, there are seven levels of food security of the population in Ukraine, which is determined by the indicator of the daily energy value of a human diet.

Kundeeva (2013) recommends to determine "the indicator of the overall physical availability of food", according to the criterion for assessing the level of food security, to assess the state of food security (levels of food security), and also, it is based on the actual state of the country's economy (unbalanced agricultural development and significant property stratification of the population) and the availability of statistical data.

The idea of evaluating food security on one indicator is attractive, but it is suitable for determining changes in the long-term dynamics or ranking of regions of Ukraine, that is, as part of a more complete monitoring of the state of food security.

Artimonova (2016) Error! Reference source not found.argues that "the goal and direction of food security are different for each level, but there are several characteristics of food security that are acceptable for assessing food security at all levels: ensuring food security; food security is ensured; unsafe food security; food disaster".

In our opinion, some of these characteristics are controversial regarding the levels of food security of the household and the individual. In particular, for the consumer, the country of production is not important if the price and quality of the product meet the requirements of the individual. The same applies to the characteristic features identified by the author as "secure food security" for a particular person, the level of consumption above the minimum necessary for physiological activity cannot be a determining indicator, since an important indicator is not the number of calories, but their quality. If consumer demand for food is met mainly through own production, this means certain restrictions for a particular person concerning her personal preferences. Every country has certain restrictions in the range of production of food products, as well as the formed food stocks, which take into account a specific list of basic products, but they do not provide the personal needs of each individual.

At the same time, we agree with the author's statement that "only the integral functioning of all levels of food security – the food security at the individual, national and international levels – will allow achieving positive qualitative changes in the provision of food products to people from all over the world, region, city and the individual person." **(Artimonova, 2016)**.

Regional level

Most scientists propose to assess food security at the regional level in the same manner as at the state level (**Error! Reference source not found.** According to the results that we substantiated, the necessity of taking into account the stability criterion of the food system of the region is determined, which requires a deeper investigation of this issue.

Piskunova and Osipova (2015) believe that "the indicator of the level and structure of per capita consumption of food can be the most informative and general indicator of the state of food security of the region."

Another group of authors **(**Error! Reference source not found.**)** propose to define an "integral indicator of food security in the regions of Ukraine": this indicator is based on the original approach, because there are less differentiation and closer interactions between the studied objects. For example, the grain stock is not taken into account, because at the regional level, the urgent transportation of grain in extreme situations is much less costly, both material and time. Consequently, only the stock of national reserves is of key importance.

Household and individual levels

The issue of food security at the household level became relevant when it was found that adequacy at the aggregated level could not ensure adequate nutrition at households or individual level. That is why the United Nations Special Committee proposed the definition of food security through the availability of food for households. Food security is provided when households have access to safe food in the amount necessary to ensure a healthy lifestyle for all its members (adequate in terms of quality, quantity, and cultural traditions) and when there is no excess risk of

loss of such access **(**Error! Reference source not found.**)**. **Error! Reference source not found.**

To establish the degree of satisfaction of personal needs through surveys, but the results will be reliable only for a certain period. On the other hand, it is fair to assert that, in other equal conditions, in the modern world, the level of income determines the level of access of a person to any material goods, including food.

Golikova (2014) believes that "food security at the household level is linked to the income of individuals." **Error! Reference source not found.**The low-income level of the population leads to the fact that price fluctuations directly affect the food situation of the poorest layers not only in the city but also in the countryside. In several countries, owners of small plots of land are frequent buyers of food, they do not provide themselves with products at the expense of their plots.

However, judging from the list of goods and services that are included in the calculations in determining the subsistence minimum, in many countries, under this term, they understood no longer the minimum need. As **Sokol (2012)** noticed, "this is no longer the subsistence minimum in its original treatment as the poverty line, but the income reflecting the minimum rates of consumption of the employee in a given period, taking into account the state of the economy." **Error! Reference source not found.**

Indicators of economic accessibility and differentiation of the cost of food by social groups become more important at the levels of the household and the individual. However, the analysis and interpretation of these indicators should be made more thoroughly according to the proposed Methodology for defining key indicators for food security. In Japan, a poor household is considered to be a family whose share of food expenditure exceeds 35% **(Nud, 2013)**. **Error! Reference source not found.**By comparing the data with such threshold values, the researcher will always have false data about the actual state of affairs. Analysis of the indicator of the differentiation of the cost of food by social groups following the Methodology for defining the main indicators of food security in the "general mass" will not allow to establish dependencies and identify the problematic aspects of household nutrition. Such an approach does not take into account the culture of food and the needs of the family, depending on their place of residence.

Scientific hypothesis

In our opinion, from a methodological point of view, the construction of a system of food security indicators should take place through the definition of the criteria for the functioning of the system. Such an approach ensures the interconnection and interdependence between the nature, objectives, and monitoring of food security at any level.

MATERIAL AND METHODOLOGY

The theoretical basis of the research is the fundamental provisions of the formation of the food security system, the modern economic theory, which defines the goals and patterns of sustainable development of the world and Ukraine, scientific works of domestic and foreign scientists on issues of food security, state administration and legislative regulation of this problem.

The methodological basis of the research is the dialectical method and general scientific and special methods of scientific knowledge. In the process of research, the following methods of economic research were used: abstract-logical (the formation of theoretical generalizations and conclusions); historical (in the study of the evolution of the formation of terminology); monographic (the study of best practices on food security issues); system analysis and synthesis (formation of a system of food security criteria based on existing problems); terminological analysis and operationalization of concepts (research of conceptual approaches to the interpretation of the concepts of "food security", "components", "criteria" and "indicators").

RESULTS AND DISCUSSION

Critical analysis of scientists' approaches to the definition of the system of food security criteria allowed to establish features of their formation in the national science (**Figure 1** – "Problem" and "Consequences"). The author's suggestions for solving these problems, including improving food security, can be summarized (**Figure 1** – "Proposals").

Therefore, when developing a system of food security indicators, we should point out the levels at which the monitoring and regulation process is conducted: global, national, and regional levels, household, and individual levels.

National level

In general, the system of indicators, approved by the Methodology for determining the main indicators of food security, meets the dimensions and criteria for food security.

At the same time, the defined system does not fully assess the criterion of implementation of effective agricultural trade policy of the state, which is the determining determinant of the national level. To ensure the objectivity of monitoring and interpretation of the obtained results, it is expedient:

(1) Include a list approved by the Methodology for defining the main indicators of food security, adding them to the system of indicators of food security assessment at the national level:

- the level of cereal stocks at the end of the period, the percentage of consumption;
- share of sales of imported food products through the trading network of enterprises, percentages;
- grain production per person per year, tons;

(2) The calculation of these new indicators should be carried out following the provisions of the Guidelines for the calculation of the level of economic security in Ukraine;

(3) Set the value of the marginal level of the indicators:

- the level of cereal stocks at the end of the period, the percentage of consumption – 100 percent;
- the share of sales of imported food products through the business network of enterprises – 25 percent;

(4) grain production per person per year – 0.8 tons **(Figure 2)**.

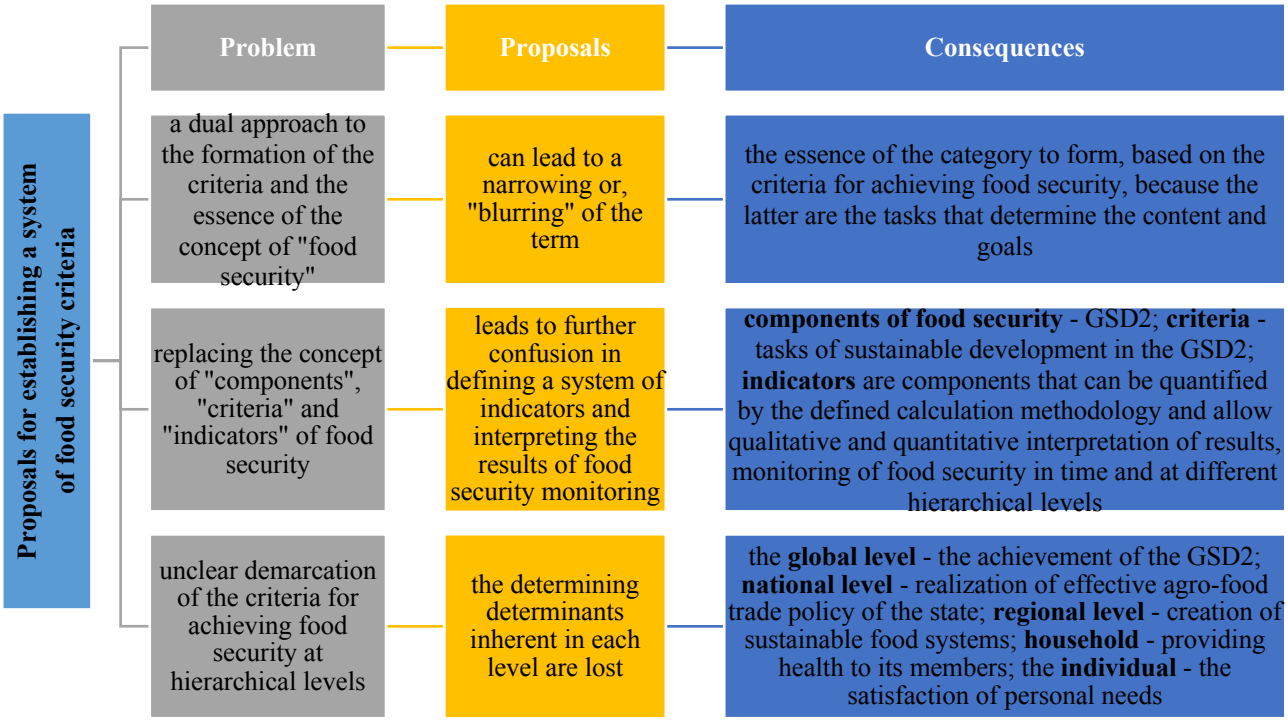

Figure 1 Proposals for establishing a system of criteria for ensuring effective food security management based on existing problems. Note: Source – Authors' work.

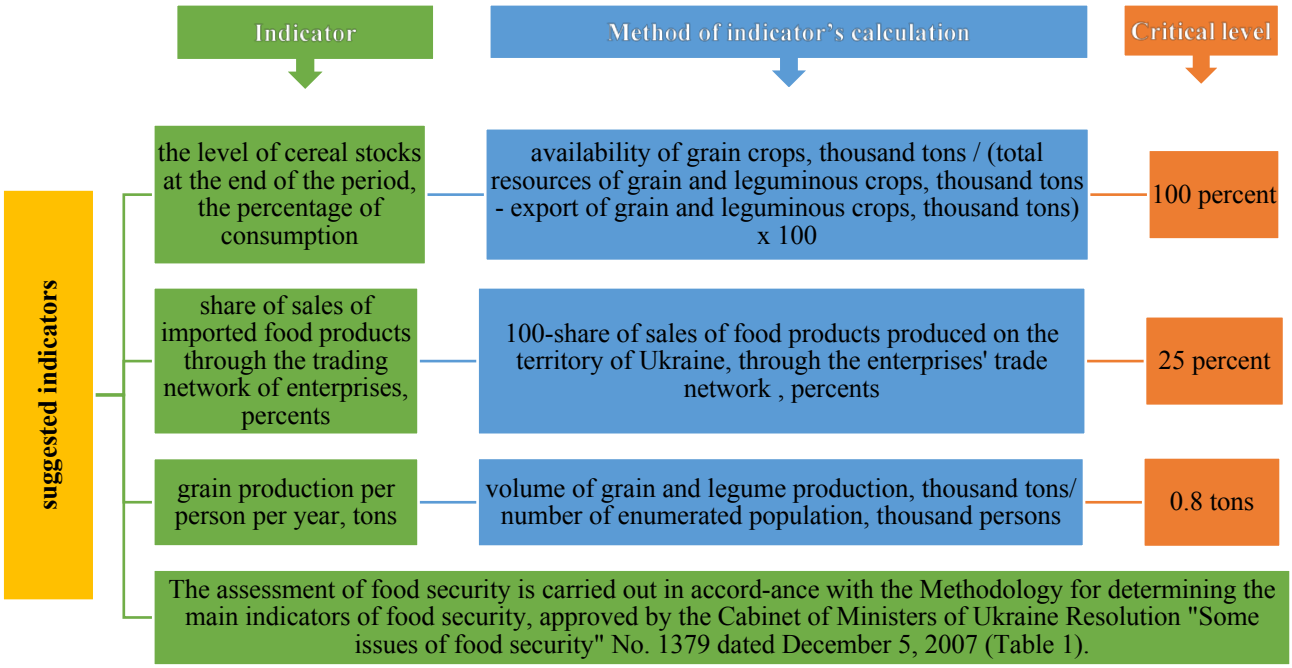

Figure 2 Proposals to improve the system of indicators to ensure effective food security management at the national level. Note: Source – Authors' work.

Regional level

Taking into account the above mentioned, there is a need for monitoring and assessment of food security at the regional level by carrying out calculations of the regional rating (ranking) of each indicator and calculating the overall rating in general for all indicatfollowingwith the Methodology for monitoring and evaluating the effectiveness of the implementation of the state regional policy (Error! Reference source not found.).

The ranking is conducted by comparing the deviation of the values of the indicators for each specific region from their best values by region for the corresponding (reporting) period and the corresponding ranking of regions from 1st to 24th place. The rating assessment is based on the calculation of relative deviations of the indicators of each region from the maximum and minimum values of such indicators of other regions by the formula:

$$R_j = \sum_{i-1}^{n} \frac{x_{maxi} - x_{ij}}{x_{maxi} - x_{mini}} + \sum_{i-1}^{n} \frac{x_{ij} - x_{mini}}{x_{maxi} - x_{mini}}, \quad (1)$$

Where: R_j – amount of the rating estimates for a particular region for each of the indicators that characterize a particular year; x_{ij} – a value of the i-th indicator in the j-th region; x_{maxi} – maximum value of the i-th indicator; x_{mini} – minimum value of the i-th indicator.

The second part of the formula is used to evaluate the indicators, which increase positively (for example, the volume of realized industrial output per capita), the first part – to assess the indicators, which increase the negative value (for example, the amount of unpaid wages).

Determination of the average arithmetic value of the sum of rating assessments of a specific region for all indicators of the annual evaluation, which characterize a separate line of activity, is carried out by the formula:

$$R_{cpj} = \frac{R_j}{n}, \qquad (2)$$

Where: R_{cpj} – an average arithmetic sum of ratings of a specific region for all indicators of a particular year; n – the number of indicators used for the calculation for a particular direction.

Due to the results of calculations, the integral rating is determined as the average arithmetic mean of the sum of rating estimates of a particular region for all years by the formula:

$$I_j = \frac{\sum_1^m R_{cpj}}{m}, \qquad (3)$$

Where: I_j – an average arithmetic sum of ratings of a specific region for all years; m – number of years for which the calculation was made.

The criterion of stability of the food system of the region is characterized by indices of production and consumption of the main types of foodstuffs, branching out of the retail chain, as well as a system for reducing, processing and subsequent use of food waste.

Official statistics do not provide information according to the latest criterion, to perform calculations of the ranking of regions with levels of food security, we propose the use of the 9 indicators **(Figure 3)**.

In general, the scheme for researching at the regional level will include six stages **(Figure 4)**.

Household and individual levels

In our opinion, it is advisable to apply the approaches proposed by Golikova K.P. **(**Error! Reference source not found.**) Error! Reference source not found.**to assess the food security at the household level, and Mostenska T.G. **(**Error! Reference source not found.**). Error! Reference source not found.**

The first approach ensures the objectivity of the analysis of the economic availability of products by comparing: the actual consumption of food with a consumer basket and rational standards; ratio and share of food costs in total costs and subsistence minimum. The second approach allows us to establish dependencies and identify the nutritional aspects of households depending on their place of residence.

In respect that neither the first nor the second approaches make it possible to characterize the health of household members as the main criterion for food security at the household level, therefore, in our opinion, to carry out a comprehensive assessment of food security at the household level, it is expedient to analyze the indicators of the expected life expectancy at birth (years) and the rate of infant mortality (deaths of children under one year per 1,000 live births). Even more problems, regarding the assessment of food security at the level of the individual, occur as follows: on the one hand, the main criterion for achieving it is the degree of satisfaction of personal needs; and on the other hand, it is necessary to comply with the principle of state administration of personal interests' subordination to the national ones, after all, eventually, only such an approach can ensure the rights and freedoms of everyone.

The hypothesis of the study is the assumption that there is a significant overproportion in the level of consumption of food products by different categories of households as well as an impact of the economic availability of products upon this misbalance. Based on this thesis, it is proposed to evaluate the level of food security of households in terms of by households' categories, based on the main indicators of food economic availability and consumption **(Figure 5)**.

There are discussions around the issue of food security indicators. In general, the proposals of domestic scientists regarding the improvement of the food security assessment system are based on the official Methodology for defining the main indicators of food security **(**Error! Reference source not found.**)** and are mainly concentrated in five areas:

(1) the formation of a system of indicators – the establishment of a list of indicators, their subordination to the criteria, the reduction or increase of their number compared with the approved Methodology for determining the main indicators of food security – were studied by **Banakh (2016)**Error! Reference source not found.**, Stezhko (2014), Ogluzdin (2010)**;

(2) improvement of the methodology for calculating the individual indicators that were proposed by the Methodology for determining the main indicators of food security, or – absolutely new indicators – were investigated by **Grishova and Kryukova (2014), Stavytsky and Prokopenko (2014)**;

(3) systematization of indicators at different hierarchical levels – was studied by **Artimonova (2016), Piskunova and Osipova (2015), Golikova (2014), Sokol (2012), Mostenska (2015)**;

(4) finding out of the optimal value of the normative (optimal, threshold and boundary) indicator level – was investigated by **Grishova and Kryukova (2014), Kochetkov and Markov (2002), Nud (2013), Kundeeva (2013)**;

(5) borrowing methodologies for assessing the food security of others (mainly post-Soviet) countries – were investigated by **Bekenov (2003), Volchenko (2013)**.

At the same time, national scientists did not agree on the construction of a unified system of indicators as part of the management of food security at different levels.

The world's scientific community also does not offer the unified indicators' system for food security monitoring at various levels, only for global one, whose indicators' system was developed by **FAO (2019a)**.

Figure 3 Suggestions for improvement of the system of indicators to ensure effective food security management at the regional level. Note: Source – Authors' work.

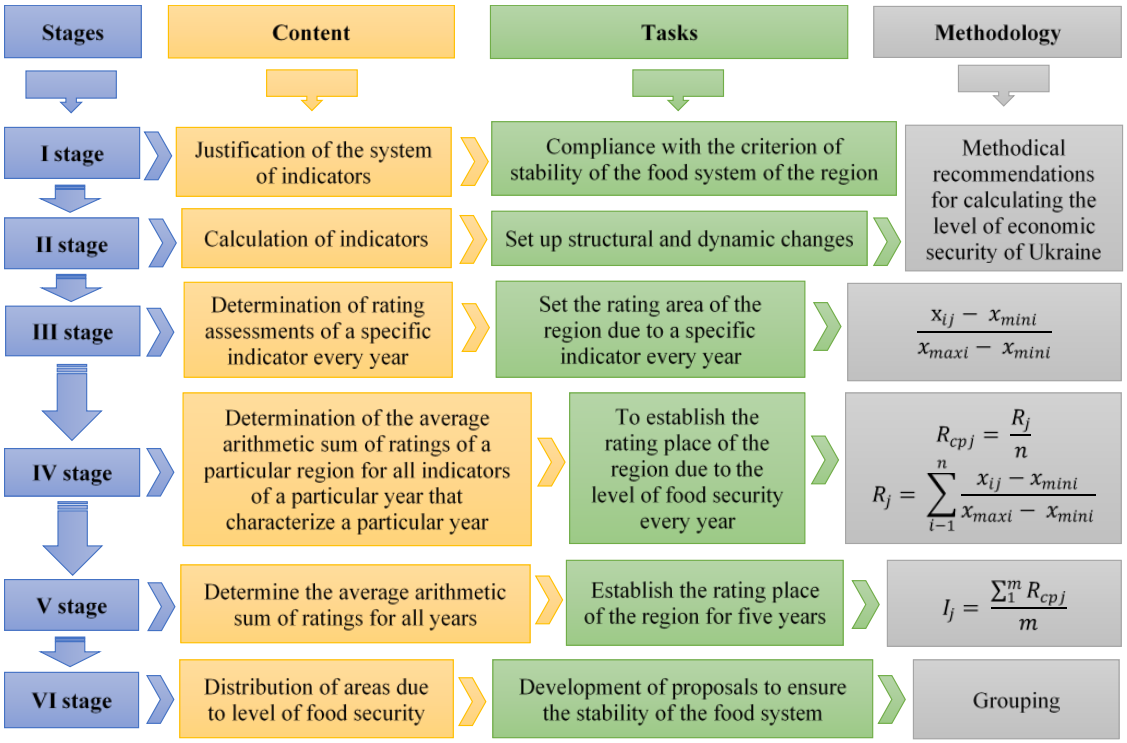

Figure 4 Methodological framework for monitoring and assessing to ensure the effective management of food security at the regional level. Note: Source – Authors' work.

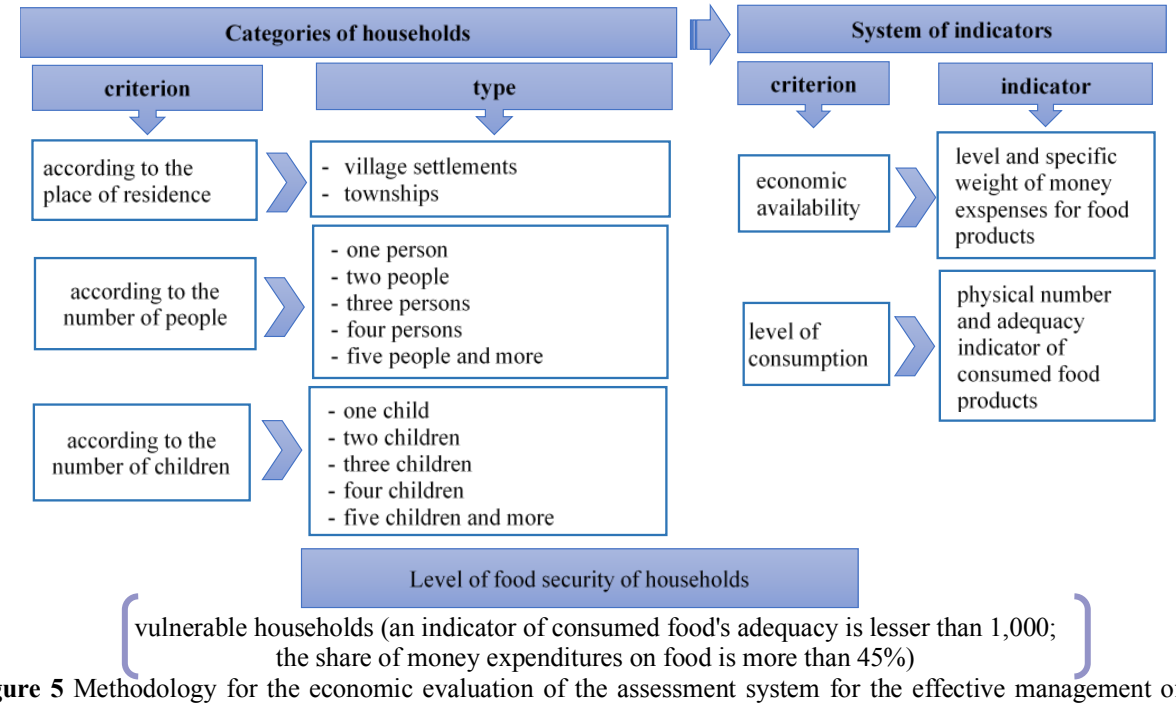

Figure 5 Methodology for the economic evaluation of the assessment system for the effective management of food security at the household level. Note: Source – Authors' work.

Table 2 The system of indicators and food security margins.

The system of indicators	Monitoring and evaluation methodology	Margin level
Global level		
FAO's system of indicators	FAO's monitoring and evaluation methodology	Threshold levels set by FAO
National level		
The system of indicators following the Methodology for determining the main indicators of food security	Methodology for determining the main indicators of food security	Threshold levels indicated in the Methodology for determining the main indicators of food security
Grain stock levels at the end of the period, percentages of consumption, %	Methodical recommendations for calculating the level of economic security of Ukraine	100 percent
Share of sales of imported food products through the company's trade network, %		25 percent
Grain production per capita per year, t		0.8 tons
Regional level		
List of 7.2-7.8 indicator due to the Guidelines for the calculation of economic security of Ukraine	Methodology for monitoring and evaluating the effectiveness of the implementation of the state regional policy	Minimum and maximum values of indicators (regional ranking for each indicator and overall rating in general for all indicators)
The ratio of trading places on the food market to the general population, ed. per 10000 people		
The ratio of the size of space for the retail stores to the total population, m2 per 1000 people		
Household-level		
Indicators proposed by Golikova K.P. and Mostenska T.G.	Approaches proposed by Golikova K.P. and Mostenska T.G.	Establishing positive or negative changes in dynamics
Expected life expectancy at birth, years	Methodological statements on the statistical analysis of the natural population movement	
Infant mortality rate (death of children under one-year-old) per 1,000 live births		
Individual-level		
System of indicators, characterizing the degree of satisfaction of personal needs	questionnaire survey	Satisfaction or dissatisfaction of the needs

Note: Italics font – suggestions by the authors for improving the system. Source – Authors' work.

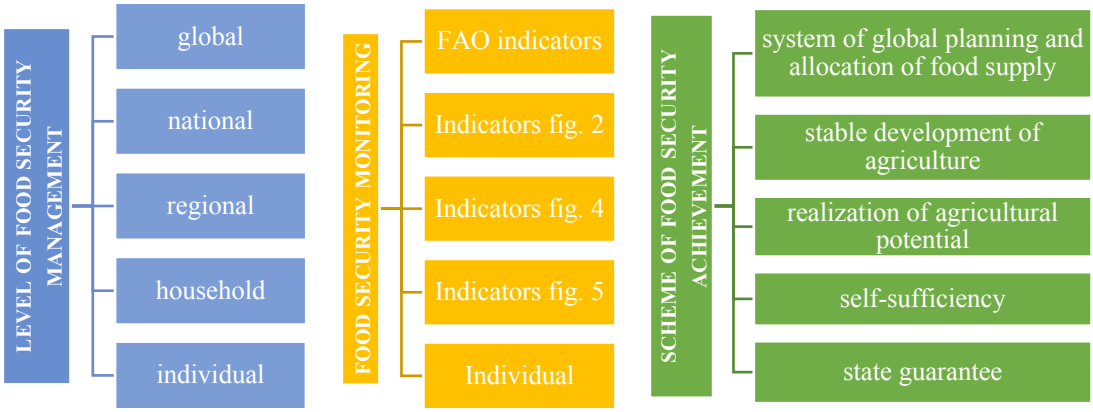

Figure 6 The place of indicators in the food security management system at different levels. Note: Source – Authors' work.

The proposed system solves a range of problems that currently exist: firstly, in the domestic monitoring system, there are developed indicators only at the national level; however, we proposed system that covers all levels; and secondly, the criteria underlying the national food security monitoring system do not meet the SDGs; instead, our proposed system of indicators is based on the need to achieve the SDGs2.

The developed system of indicators and margins of food security ensures monitoring and evaluation of the achievement of the criteria of the respective levels (global, national, regional, households, and individuals) based on the actual official information provision of the processes of food security according to the SDGs2.

The proposed system of indicators allows monitoring of food security in a systematical and integrated way, at various hierarchical levels, which enables the quality management of food security taking into account specific features and needs at each level **(Figure 6)**.

Further research will be aimed at making the strategic framework and mechanism for ensuring food security at various hierarchical levels, taking into account the monitoring results of the proposed methodology.

CONCLUSION

The formation of theoretical and methodological guidelines and methodological recommendations for the development of a system of food security indicators requires further systematic research on its content-based basis as a component of food security management at different levels in the context of the implementation of the concept of sustainable development goals 2016 – 2030.

Based on the results of the conducted research and the scientific and theoretical positions of the epistemological content of the category of the concept of "food security", taking into account the criteria of food security, formation at different hierarchical levels and methodological aspects of its monitoring, we propose:

(1) to use the FAO's system of indicators to assess global food security. It makes no sense to develop a new system that requires complex calculations in all countries of the world (because the global level has the same information – comparisons across different countries, continents, continents, etc.) when FAO outlines all indicators and reports on the state of food security in the world in open access;

(2) to assess food security at the national level, we should use the system based on the indicators of the Methodology for defining the main indicators of food security, taking into account the indicators characterizing the level of the country's agricultural and food trade policy;

(3) to use a system based on the indicators of the Methodological recommendations for assessing food security at the regional level, for calculating the level of economic security of Ukraine, taking into account indicators that characterize the stability of the food system;

(4) to assess food security at the household level, use the technique proposed by **Mostenska (2015)**, taking into account indicators that characterize the health of household members;

(5) the assessment of food security at the individual level is currently only possible through the application of a questionnaire, where attention should be focused on the analysis of indicators characterizing the degree of satisfaction of personal needs.

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PATIN (*PANGASIUS HYPOPHTHALMUS)* FISH PROTEIN CONCENTRATE ALTERS INSULIN-LIKE GROWTH FACTOR (IGF)-1 AND IGF BINDING PROTEIN (IGFBP)-3 LEVEL OF SPRAGUE DAWLEY NEONATE RATS-INDUCED MALNUTRITION

Annisa Zikra Abdullah, Samnil Astuti Fitri, Muflihatul Muniroh, Tri Winarni Agustini

ABSTRACT

Malnutrition is caused by inadequate protein intake and affects growth factor. High protein from patin (*Pangasius hypophthalmus*) fish is a well-known protein source. This study aims to investigate the effect of patin fish protein concentrate (PFPC) in the IGF-1 and IGFBP-3 level of Sprague Dawley (SD) neonate rats-induced malnutrition. Thirty male SD neonate rats were divided randomly into five groups, namely normal control (K1), malnutrition control (K2), malnutrition with PFPC 13.26 mg.g^{-1} body weight (BW)/day (X1), malnutrition with PFPC 19.89 mg.g^{-1} BW/d (X2), and malnutrition with casein supplement 13.26 mg.g^{-1} BW/d (X3). K1 received a standard diet, while the others received a low 8% protein diet (L8PD) since those were born until 21 days. The standard diet was refed for all groups during the intervention (14 days). IGF-1 and IGFBP-3 levels were measured by ELISA. Normal data were analyzed by using One-way ANOVA which then was followed by post-hoc Bonferroni. Meanwhile, the others were analyzed by Kruskal Wallis and followed by Mann-Whitney U-test. Spearman test was used for correlation. PFPC contained 81.07% of protein, 4.08% of fat, 7.24% of moisture, 2.77% of ash, and 4.83% of carbohydrate. These contents had affected the growth factor. As a result, in the PFPC intervention, IGF-1, and IGFBP-3 levels (p <0.05) were decreased, while the controls were increased. The decreased values were shown in IGFBP-3 levels (p <0.05) while the increase was shown in both controls. On the other hand, the increase in body weight was shown in all groups, including control ones. A strong correlation was found between IGF-1 and IGFBP-3. PFPC has additional value on repairing malnutrition that is the best dose in effecting IGF-1 dan IGFBP3 levels is 13.26 mg.g^{-1} BW/d.

Keywords: FPC; Malnutrition; IGF-1; IGFBP-3

INTRODUCTION

Malnutrition is generally caused by restricted dietary intakes and ended with poor linear growth even in early life **(Kartini, 2019; Aheto, 2015)**. Globally, undernutrition conditions have been caused by the mortality of 30% of children **(Blossner and De Onis, 2005)**. The prevalence is still high in some developing countries in South East Asia such as Indonesia **(UNICEF, WHO, and World Bank Group, 2018)**.

Malnutrition is caused by a lack of nutrients intake, such as a protein restriction diet **(Kartini, 2019; Saunders, 2011; Gibson, 2005)**. Malnutrition, especially in the golden age, can cause growth faltering, such as stunting. Besides is also has a reciprocal relationship with infections that lead to systemic inflammation **(Bartz, 2014; Guerrant, 2008; Lunn, 2007)**. Consequently, this condition results in the disruption of the body's hormonal function, including disruption of growth hormone (GH). Furthermore, the disruption also affects the role of GH in releasing growth-related substances in the hypothalamus-pituitary-GH axis interaction. Malnutrition will also affect the mechanism in producing IGF-1 and IGFBP-3, the binding protein which is the principle binding protein of IGF-1 **(Deboer, 2017; Bartz, 2014)**.

IGF-1 and IGFBP-3 are produced in the liver. They have a function in mediating the protein metabolism and controlling the growth factors which are accumulated in somatic cells **(Guntur and Rosen, 2013; Skottner, 2012; McDonald, 2007; Laron, 2001)**. Their production is a form of GH secretion and even shows a resistant condition **(Misra, 2003; Hintz, 1978)**. Decreased IGF-1 values are shown in acute malnutrition, while IGFBP-3 in chronic one. Both of them have special functions in several metabolic conditions **(Gupta, 2011; Hoppe, 2004)**. Several studies on subjects with severe acute malnutrition, even normal, showed a decrease **(Kartini, 2019; Hoppe, 2004; Misra,**

2003). Consuming high protein intake can improve this condition especially the function of IGF-1 and IGFBP-3.

Fish protein concentrate is a product resulting from the relieving moisture and fat content. Bioactive compounds and peptide contained in fish have benefits to control growth **(Kundam, 2018)**. Patin (*Pangasius hypophthalmus*), a freshwater fish, is a tropical fish that can live in many countries in Southeast Asia such as Indonesia. Many fisheries technologies can maintain and protect the fish protein from damaged, such as by making fish protein concentrate. The fish protein intervention studies are well-known studies and affect many pre-clinical and clinical indications **(Nobile, 2016)**. With 80% protein reaches of PFPC and has greater digestibility, amino acids from patin fish, especially lysine and leucine, are more absorbed by the body than other protein sources, such as meat and poultry protein **(Pratama, 2018)**. Discussing the high protein study and its effect on linear growth and malnutrition, high protein intake has shown its effects in increasing IGF-1 in boys and increasing IGFBP-3 of premature infants **(Hoppe, 2004; Smith, 1997)**. This study aims to investigate whether the intervention of patin fish protein concentrate affects the IGF-1 and IGFBP-3 levels of Sprague Dawley (SD) neonate rats-induced malnutrition.

Scientific hypothesis

There are various doses of patin fish protein concentrate that affect insulin-like growth factor (IGF)-1 and IGF binding protein (IGFBP)-3 levels of Sprague Dawley neonate rats-induced malnutrition.

MATERIAL AND METHODOLOGY

Preparation of patin fish protein concentrate

Patin fish were bought at fishpond in the Ambarawa sub-district, Semarang Regency, Central Java Province, Indonesia. The selected patin fish as those that have a good condition as shown by fresh patin that has just been captured, weighs about 1 kilogram for one fish, has fresh flesh, has gills that are still red, the color does not change and odorless. Then the patin fish were filleted and cleaned. The filleted fish were ground using a food processor with the addition of salt (0.5%) and $NaHCO_3$ (1.5%). Next, fish pasta was steamed for 30 minutes. The steamed pasta was then pressed and extracted by 96% food-grade ethanol with a 3:1 patin ratio. The extraction was carried out twice to fully relieve the moisture and fat content. The extracted fish was then dried at a temperature of 40 ^oC and mixed until refined size (60 mesh). The PFPC was wrapped in food-grade silica gel and stored in the bottle at 4 °C **(AOAC International, 2006)**.

Table 1 Ingredient compositions (g/kg) of low 8% protein diet (L8PD) fed to malnutritiomn-induced rats.

Ingredients	L8PD (g)*
Casein purified high nitrogen	80
Corn starch	780
Cottonseed Oil	100
Salt mixture U.S.P XIV	40
Total	1000

Note: * L8PD for K2,X1,X2, and X3.

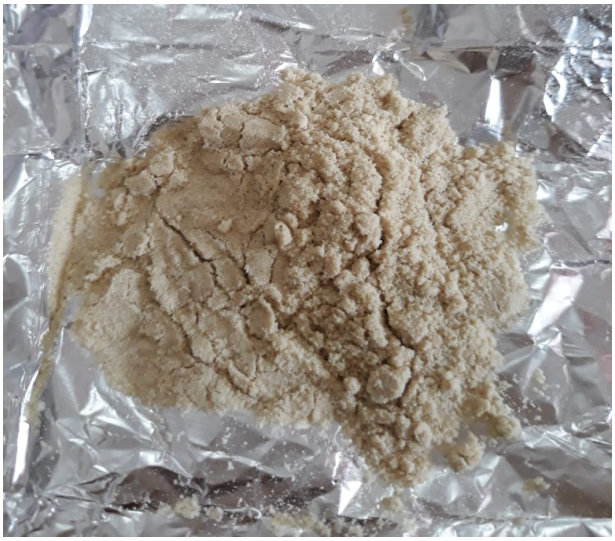

Figure 1 Patin Fish Protein Consentrate.

Proximate analysis of patin fish protein concentrate

The proximate analysis includes moisture, protein, ash, fat, and carbohydrate content (by difference) **(AOAC, 2005)**.

Research design and experimental-animals

This research was a true-experiment study with a randomized pre-post test and has a control group design. The animal used was male Sprague Dawley rats, aged 21 days induced with a low 8% protein diet (L8PD), except health control. The acclimatization was held in the laboratory of Gadjah Mada University, Yogyakarta. The rats were placed in each group and numbered. They were placed at a regulated temperature (21^oC) and a clean cage. They were given ad-libitum water during the experiment. Animal care in the laboratory was carried out in accordance with the animal Laboratory Guideline from the Central Laboratory for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta.

Thirty rats were divided into five groups, namely groups of normal control (K1), malnutrition control (K2), malnutrition with PFPC 13.26 $mg.g^{-1}$ BW/day (X1), malnutrition with PFPC 19.89 $mg.g^{-1}$ BW/d (X2), and malnutrition with casein supplement 13.26 $mg.g^{-1}$ BW/d (X3) **(Wong, 2019)**. The malnutrition-condition was induced since 0 - 21 days old of neonate rat or through maternal L8PD **(Table 1)**. After 3 days of acclimatization, the IGF-1 and IGFBP-3 were examined before the intervention. The intervention group was treated with the CMC-Na suspension method for 14 days. Body weight was measured every week since the animals were born until the interventions were ended. All of the groups were fed with Comfeed II standard-diet (CSD) during the intervention period. The blood sample was taken again at the end of the intervention through the retroorbital plexus. Blood samples were collected in a centrifugation tube and centrifuged 4000 rpm in 15 minutes. IGF-1 and IGFBP-3 levels were analyzed by ELISA **(Kartini, 2019; Alishahi, 2013)**.

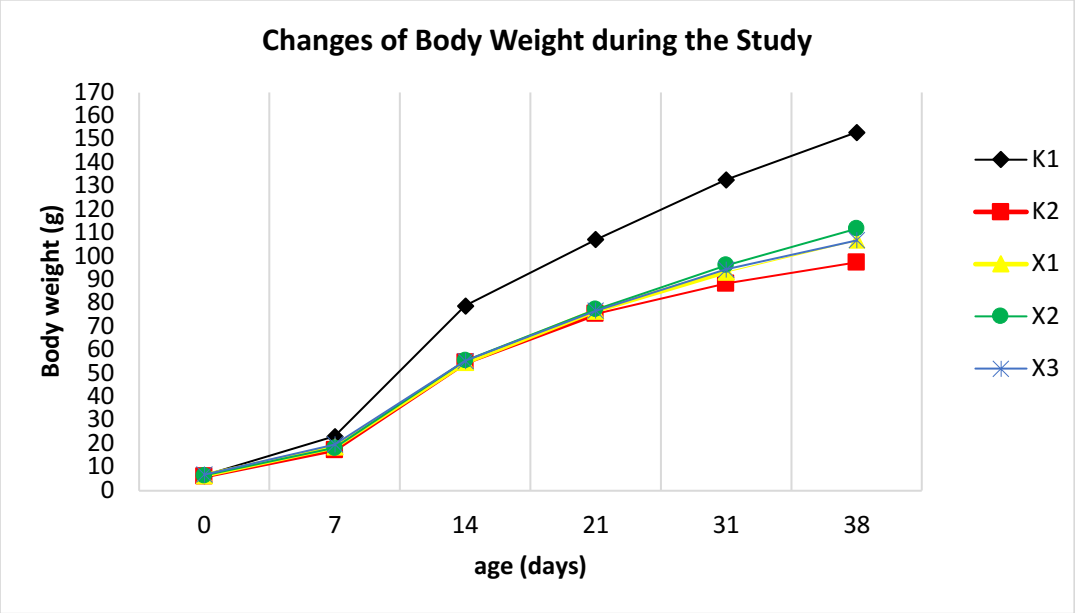

Figure 1 Changes of body weight of neonate rats during the study. Malnutrition was induced from day 0 to 21st by giving low 8% protein diet (L8PD). PFPC intervention was started from day 24th to 38th. Three days acclimatization was done from day 21st to 24th.

Ethical declaration:

This study was approved by the Health Research Ethics Commission of the Faculty of Medicine Diponegoro University Semarang through ethical clearance No. 131/EC/H/KEPK/FK-UNDIP/X/2019.

Statistical analysis

Results were expressed as mean ± SD (for normally distributed data), otherwise, it was expressed as median (min-max). Statistical difference was analyzed by using a one-way analysis of variance (ANOVA) followed by post hoc Bonferroni for normally distributed data, otherwise, the Kruskal-Wallis test followed by Mann-Whitney-U-test was used. The software used was SPSS software version 22 (SPSS Inc, Chicago, IL, USA). Spearman's correlative test was used to analyze the relationship between variables. Statistical analyses were done by the computer. The differences and correlations were considered significant at *p*-value <0.05 and 95 % confidence intervals. The strength of the correlation was determined by the *r-value*.

RESULTS AND DISCUSSION

Related data about PFPC was shown as proximate analysis **(Table 2)**. The other data about intervention processed was obtained from 30 Sprague Dawley-neonate rats, which were divided into each group consisting of 6 neonatal rats. The experimental animals used before intervention had an average body weight of 76.58 grams for the malnutrition group and 106.5 grams for the healthy control (data not shown).

Wilcoxon test carried out on the body weight before and after the intervention, showed that the intervention of PFPC increased the body weight in the all-treatment-group as shown in **Table 3** and **Table 4**. Healthy-control-K1-group has experienced a significant increase in BW ($p = 0.026$). Statistically, the difference was also found on the pre-post intervention change (Δ) of neonate rats-BW among all groups (Kruskal Wallis test; $p = 0.001$). Meanwhile Mann-Whitney U- test was also performed on two groups. The Δ-neonate rats-BW of all-intervention-malnutrition-group were higher than malnutrition-control-K2-group and this result was significant (X1 and X2 had the same *p*-value; $p = 0.004$). The Δ-neonate rats-BW of the X1-group was not different from the X3-group ($p = 0.868$). This finding demonstrated that PFPC can increase body weight which is higher in malnutrition neonate rats. Besides, this finding also showed that the ability of PFPC in dose 13.26 $mg.g^{-1}$ BW/d in increasing body weight of malnutrition neonate rats has the same ability obtained from the casein supplement. The difference in body weight obtained during the study was shown in **Figure 1**.

The IGF-1 levels decrease at the end of the intervention, and significantly different ($p = 0.028$ for X1 and X2, $p = 0.027$ for X3; **Table 4**), while K2-group experienced a significant increase IGF-1 level ($p = 0.027$). The pre-post-intervention-change (Δ) of IGF-1 levels was significantly different among five-groups (Kruskal Wallis test; $p = 0.001$). Meanwhile, the Δ-IGF-1-levels of all-intervention-malnutrition-group were different compared to malnutrition-control-K2-group (X1, X2, and X3 had the same *p*-value; Mann-Whitney U-test, $p = 0.004$). But, in suppressing of IGF-1 between all-malnutrition-treated-group, statistical analysis showed that the Δ IGF-1 in X1-group was not different from X2 ($p = 0.055$) and X3-group ($p = 0.054$). This finding indicated that the ability of PFPC in dose 13.26 $mg.g^{-1}$ BW/d was not different from the PFPC in dose 19.89 $mg.g^{-1}$ BW/d and the given of casein supplement.

Table 2 Proximate analysis of PFPC.

Nutrient Content	%
Moisture	7.24 ±0.35
Ash	2.77 ±0.07
Protein	81.07 ±0.56
Fat	4.08 ±0.18
Carbohydrate	4.83 ±0.30

Note: * L8PD for K2,X1,X2, and X3.

Table 3 Statistical analysis of body weigth, IGF-1 and IGFBP-3 (control groups).

Variables	K1				K2			
	Pre	Post	p^a	Δ	Pre	Post	p^a	Δ
BW	106.5 (104 - 112)	152.5 (149 - 158)	0.026	48 (38 - 53)	75.50 (70 - 81)	98.5 (92 - 101)	0.026	22.00 (20.00 - 23,00)
IGF-1	41.32 (39.77 - 42.53)	42.35 (40.81 - 43.22)	0.026	1.03 (0.69 - 1.38)	69.04 (67.67 - 71.11)	70.59 (68.70 - 72.14)	0.027	1.20 (0.69 - 1.72)
IGFBP-3	40.89 ±2.69	42.07 ±2.43	0.004	1.17 ±0.57	92.16 ±1.96	94.53 ±2.23	0.034	2.37 ±1.99

Note: [a] p-values were obtained by Wilcoxon test (for BW and IGF-1), paired t test (for IGFBP-3); between pre-post intervention.

Table 4 Statistical analysis of body weigth, IGF-1 and IGFBP-3 (group with PFPC and casein intervention).

Variables	P1				P2				P3				p^b	p^c	p^d
	Pre	Post	p^a	Δ	Pre	Post	p^a	Δ	Pre	Post	p^a	Δ			
BW	77 (71-80)	108 (100-109)	0.027	29.5 (28-34)	77 (73-82)	110 (107-118)	0.027	34.50 (33-36)	76.5 (75-79)	107 (104-109)	0.026	29.5 (29-32)	0.005	0.001	0.001
IGF-1	69.56 (67.67 - 99.35)	53.72 (52.51 - 54.92)	0.028	-16.01 (-44.42 - 13.77)	68.70 (66.29 - 71.11)	39.77 (34.95 - 41.15)	0.028	-29.61 (-33.75 - -25.14)	68.53 (66.63 - 69.73)	44.42 (42.87 - 45.63)	0.027	-23.59 (-25.83 - -23.07)	0.003	0.001	0.001
IGFBP-3	92.56 ±2.43	66.23 ±2.37	0.001	-26.32 ±3.23	90.59 ±2.65	49.33 ±1.23	0.001	-41.26 ±2.79	92.71 ±4.50	46.56 ±1.45	0.001	-36.15 ±3.60	0.001	0.001	0.001

Note: [a]*p*-values were obtained by Wilcoxon test (for BW and IGF-1), paired t test (for IGFBP-3); between pre-post intervention. [b]*p*-values were obtained by Kruskal Wallis test (for BW and IGF-1), One-way ANOVA test (for IGFBP-3); pre-intervention among groups. [c]post-intervention among groups. [d] Δ among groups.

The IGFBP-3 levels experienced a decrease after the performance of the intervention. The levels were significantly different (X1, X2 and X3 had the same *p*-value; $p = 0.001$; **Table 4**), while K2-group experienced a significant increase of IGFBP-3 level ($p = 0.034$). The pre-post intervention-change (Δ) of the IGFBP-3 level was significantly different among the five groups (One-way ANOVA test; $p = 0.001$). The Δ-IGFBP-3-levels of all-intervention-malnutrition-group were different compared to malnutrition-control-K2-group (X1, X2, and X3 had the same p-value; Post Hoc Bonferroni test, $p = 0.001$).

The Spearman test on all data from all rats at the end of the study showed there was a very strong correlation that was found between the variables (IGF-1 and IGFBP-3). A very strong positive correlation was observed between IGF-1 and IGFBP-3 (r=0.861). This correlation was less weak before PFPC intervention began (data not shown).

Malnutrition can be ended with a linear growth disruption. Restriction of protein intake during the lactation period affects the disruption of long term linear growth **(Cherala, 2006; Zambrano, 2005)**. We examined that the decreasing value of IGF-1 levels in the malnutrition-treatment-group after the intervention was caused by an inflammatory response. Systemic inflammation which occurs due to protein restriction during early life has not been repaired by early re-feeding.

Inflammation is a possible indication in the intervention group which is shown through the decreasing serum IGF-1 levels in neonatal rats. Indications of inflammation are thought to occur due to the placement of each group of neonate rats in one cage. The same result was also found in other previous studies showing the effect of interleukin 6 (IL-6) inflammatory biomarkers on IGF-1 descent. The decrease of IGF-1 is related to the mitogenic function of IGF-1 thereby inducing a decrease of its concentration in cell proliferation **(Chen, 2009; Lann and LeRoith, 2008; Jenkins, 2000)**.

These indications of inflammation due to chronic malnutrition are also associated with GH resistance response, which modulates gluconeogenesis **(Hawkes and Grimberg, 2015; Fazeli and Klibanski, 2014; Difedele, 2005; Turner, 1976)**. This condition can be attributed to the increase of GH and growth hormone receptor (GHr) which modulates glucose homeostasis as indicated by an increase in body weight. However, in the mechanism of the endocrine system, the GH resistance condition causes a decrease in IGF-1 and IGFBP-3 concentrations **(Martins, 2017; Liu, 2004; Hefferman, 2001)**.

Other previous studies have assessed that serum IGF-1 levels can be assessed at four weeks of intervention. Meanwhile, this study assessed the IGF-1 in two weeks' intervention. This is also supported by the insignificant assessment of protein synthesis which ended with a disruption of metabolism especially the disruption of IGF-1 synthesis **(Caregaro, 2001)**. The disruption of synthesis of the pituitary-GH-IGF1 axis is a major factor in the decrease of serum IGF-1 levels after re-feeding. The chronic malnutrition intake which has experienced by the subject suppresses the key components of IGF-1 and indicates impaired its production in the liver **(Zamboni, 1996)**. This condition will provide IGF-1 negative feedback to the hypothalamus which results in an in increasing GH release. The decrease of IGF-1 in this study can also be related to the physiological response of IGF-1 which uses amino acids together with its pro-insulin structure, despite the subjects has been re-feed with high protein intake **(Bonefeld and Moller, 2011)**. The other inhibitor that can be related to IGF-1 activity is adjusted with the IGFBP sub-type on an individual subject and the protease of the binding protein which is modulated by the level of their affinity **(Kamycheva, 2012; Ferry, 1999)**.

Decreasing serum IGFBP-3 levels can cause disrupt IGF-1 modulation. IGFBP-3 is the principal binding protein that modulates the action of IGF-1 as a growth factor. Disturbances that are caused by protein restriction and are not properly repaired realized that the pituitary-GH-IGF axis binding protein has a reciprocal effect **(Bang, 1994)**. However, in this study, we found a negative relationship where there was a decrease in IGFBP-3 levels after the intervention. Previous studies that showed a decrease in IGFBP-3 proteolysis activity can make the binding protein of IGF-1 as a trigger of low levels of IGF-1 targets. The potential of IGFBP-3 in proteolysis depends on its post-translational modification which has a great impact on IGF-1 **(Rosen and Yakar, 2020)**. Interestingly, the proteolysis mechanism of IGFBP-3 can occur with unknown protein in the extracellular matrix besides IGF-1. This condition exactly affects the decreasing IGF-1 level **(Vorwerk, 1998; Bereket, 1995; Bang, 1994)**.

Proteolysis is the most possible mechanism in this study which has a function to reduce serum IGFBP-3 levels. Both levels of IGF-1 and IGFBP-3 which were decreased show that the mechanism most likely caused by proteolysis **(Dauncey, 1993)**. Interventions in malnutrition children by re-feeding show a decrease in proteolytic activity, but restoring energy intake in the subjects **(Zamboni, 1996)**. Decreasing the potency of proteolysis contributes to reducing the ability of IGFBP-3 to bind with IGF-1. The other reason is other competitors that can bind with IGF-1, such as IGF-1 receptors. These statements are related to IGFBP-3 which regulates IGF-1 bioactivity through non-specific proteases **(Pucilowska, 1993; Davenport, 1992)**. The action capability of this specific IGFBP-3 can even limit the bioavailability of free IGF-1 which is further regulated in the extracellular space if it is proteolysis must be done **(Rosen and Yakar, 2020)**. This research proved that IGF-1 can be modulated by one of its main binding proteins (IGFBP-3), which is supported by acid-labile sub-unit to make a binary complex.

Interestingly, other growth factors besides IGF-1 and IGFPB-3 might be involved and affected the increasing body weight of neonate rats. These growth factors show the effect of suppression on IGF-1 and IGFBP-3 but support the mechanism of other growth biomarkers which is associated with the increasing body weight **(Bereket, 1995; Bang, 1994)**. Excess of GH secretion, as the impact of its resistance condition, is stimulated by excess exactly of GHr secretion to support GH function. However, this condition reflects complete negative feedback as the result of decreasing in IGF-1 levels **(Liu, 2004; Zamboni, 1996)**. The effect of increased GHr showed in previous studies modulates the increase in somatic growth **(Liu, 2004; Hefferman, 2001)**. This hypothesis supports the results of this study. Deleting of GHr gene will affect decreasing body size **(Liu, 2004)**. In this study, malnutrition neonate rats which were re-feed with supplementation of PFPC showed a significant growth acceleration compared to the healthy control.

Malnutrition condition, which was caused by cachexia symptoms and inflicted from malignancy response, also observed a similar indication. It was shown significantly by a specific combination of high-protein intake intervention which contained leucine **(Van Norren, 2009)**. Increasing body weight is a positive effect from consuming high-protein intake which modulates the anabolism process in many cells and tissues is targeted by protein synthesis and the contribution of amino acids **(Rogers and Harper, 1965)**.

The findings in protein intervention also improve clinical symptoms due to protein restriction. Other growth factors can affect the increase in body weight of malnutrition with re-feeding **(Soliman, 1986)**. Increasing body weight can be driven by the anabolic response that is controlled by GH. The increase of GH secretion provides an alternative source of metabolism through fat mobilization and hepatic glucose production. This response has been reported in subjects without insulin resistance in basal conditions **(Smith, 1981)**.

The increase of body weight which is indicated by increased GH is also associated with carbohydrate and fat homeostasis **(Liu, 2004)**. Other studies conducted in children with marasmic and kwashiorkor showed lower serum IGF-1 levels in the marasmic group. This shows that energy intake takes the main place to process rather than the food protein process **(Underwood, 1994; Dauncey, 1990)**. Another study also observed re-feeding carried out by returning the intake of rats to the standard diet could allow that the high intake of protein would not necessarily be effectively metabolized.

Furthermore, amino acid, especially lysine and leucine which are high in PFPC products could also change weight gain. We found in this study that the PFPC contained protein amounting 80% (**Table 2**) and the PFPC in a dose of 13.26 $mg.g^{-1}$ BW/d did not have different effects from casein supplement in the same dose. These findings indicate that the ability of PFPC is no different from casein, which has purified high protein. It is expected that the PFPC has certain amino acids for gaining weight. Other studies also recognize that oral leucine supplementation stimulates muscle protein synthesis and is not dependent on insulin **(Dardevet, 2002; Anthony, 2000)**. Once again, those findings supported this study that the mechanism of decreasing IGF-1 does not affect the increase of body weight because of its function associated with amino acids

and pro-insulin **(Bonefeld and Moller, 2011; Rinderknecht and Humbel, 1978)**. Although the protein concentration in this study product was high, even exceeding the concentration of similar products from the sThe isoleucine contin PFPC is higher than white fish protein hydrolysate **(Windsor and Barlow, 1981)**. But, we did not further examine the amino acid content and their digestibility through in vitro studies.

The high protein concentration also supports the lipid and carbohydrate pool of neonate rats. Increased protein intake can affect the amount of lipids and carbohydrates stored in adipose tissue, especially white adipose tissue (WAT) **(Zhang, 2019)**. As the largest cholesterol provider, WAT can be involved in cholesterol metabolism where cholesterol can increase to high-density lipoprotein (HDL) **(Zhang, 2010)**. Besides WAT is also involved in the endocrine system which secretes protein factors, such as adipokines associated with energy regulation and inflammatory homeostasis **(Rafols, 2014; Khovidhunkit, 2004)**. From the results of this study and existing theories, they indicate that protein intake and high-fat content of PFPC products affect the function of the adiposity system and lipoprotein. This mechanism puts an end to weight gain even though there is an indication of inflammation that is shown from the decrease in IGF-1 and IGFBP-3 levels.

Limitations of this study we do not examine other growth indicators on this intervention except the body weight. Discussing another growth factor, we also do not examine another related growth factor that can relate to and support the hypothesis of this study. Inflammation is not measured in this study, as expected to exist in chronic malnutrition neonate rats. The proteolytic, which can be the most possible involved mechanism in this study is also not concerned. We did not concern about the digestible amino acid of PFPC which can be compared to other products in case of body weight gain of malnutrition rats. Other biomarkers that relate to lipid metabolism also not a concern.

CONCLUSION

The administration of patin fish protein concentrate with various doses tested, significantly increased the body weight, but decreased IGF-1 and IGFBP-3 serum level of malnutrition-induced neonatal rats. The administration of patin fish protein concentrate with dose 13,26 $mg.g^{-1}$ body weight per day is the most effective dose in increasing body weight of malnutrition-induced neonatal rats.

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PHYSIOLOGICAL STATE OF PLANTS AND QUALITY OF PLUM FRUITS GRAFTED ON THE ROOTSTOCKS OF VARIOUS STRENGTH OF GROWTH DEPENDING ON THE PLANT NUTRITION MODE

Valentina Popova, Natalya Sergeeva, Olesya Yaroshenko, Anna Kuznetsova

ABSTRACT

The influence of the nutrition mode on the physiological state, productivity, and quality of the plum harvest of the Stanley variety on the rootstocks of various strengths of growth was investigated. The fertilizer system included the intra-soil application of the complex organic-mineral fertilizer based on peat and non-root dressings combined with the "Novosil" phytoregulator. The nutrition mode of plum plants was studied by the method of gross content diagnostics of elements in leaves and fruits. The significant increase of nitrogen and phosphorus content, number of functional pigments, free amino acids, and protein synthesis, especially in plants on the rootstock Best, was established during the period of the intensive shoot and ovary growth. In the early summer, the increase of calcium content and decrease of secondary metabolite one was found against the background of a decline of growth activity in plum leaves on the dwarf and medium rootstocks. The statistically reliable positive correlation between nitrogen, potassium, calcium and their content in plum fruits on the rootstock VVA 1 ($r = 0.68$, $r = 0.74$, $r = 0.52$) and rootstock Best ($r = 0.75$, $r = 0.84$, $r = 0.61$) was identified against the background of the application of fertilizers during the period of stress influence of abiotic factors in July. The green pigment content was also increased. The decline of protein content in leaves in the trees on the rootstock Best and rootstock PK SK 1 was minimal. At that time maximum losses of shared water were in plants on the rootstock cherry plum seedlings and minimum ones were on the rootstock PK SK 1. In early August the gross content of nitrogen, potassium, and calcium in the leaves of shoots increased with the application of fertilizers. In early September the excess of the sum of amino acids in plum leaves was 26,6-30,8% while the one in plum fruits was 12.5 and 23.5%; 24.2 and 11.5%; 17.3 and 19.6% on the rootstock VVA 1, rootstock Best, rootstock PK SK 1 respectively. The nutrition mode optimization promoted increasing of plant productivity on the rootstock Best and fruit weight on the rootstock VVA 1, rootstock Best, and rootstock PK SK 1.

Keywords: *Prunus domestica* plum; rootstocks; plant nutrition mode; physiological state; productivity

INTRODUCTION

Growing mainly on the rootstock cherry plum seedlings, *Prunus domestica* plum is widespread in industrial plantings of stonecrops of Krasnodar territory located in the west part of Caucasus and Ciscaucasia. The popularity of this culture is related to the precocity and the stable yield **(Eremin et al, 2000; Eremin, Prichko, Kekhaev, 2007; Eremin et al, 2010; Eremin and Brizhinov, 2011)**. The specific humid climate conditions are necessary for the successful growth and development of plum plants because the water holding capacity of tree bark and wood is unsatisfactory **(Miletić et al, 2007)**. The coefficient of water consumption to make a unit of tree mass dry matter and harvest is high, therefore, sufficient air humidity is necessary during the period of flowering and growth of shoots **(Gnezdilova and Doroshenko, 1984; Eremin, 2000; Bogdanov, 2003)**. The area is characterized by the diversity of soil and climate conditions: from temperate continental to subtropical one. There are also almost all main soils of the temperate zone that are suitable for intensive cultivation of the fruit growing industry there. Chernozem, forest, and meadow soils are the most common – Anthrosols **(IUSS Working Group WRB. 2014)**. The species composition of chernozems varies significantly changing from North to South. The main differences are in the organic matter content and the power of the humus layer, mechanical composition, soil compaction, presence of signs of salinity. Various degrees of erosion processes, the waterlogging in winter, and the formatting of verkhovodka (superficial water) are typical for foothills soils (grocerie agroseme texture-differentiated and agronomy structural-metamorphic) **(Shishov L. L et al, 2004)**. Alluvial agrohumus hydrometamorphic soils with small power of humus layer and a wide range of nutrition

elements stock that are available for plants are prevalent in the river valleys **(Simakin, 1969; Negovelov et al, 1985; Eisert, Achkanov, Durgaryan, 1987; Lipchiu and Gagai 2014)**. At the same time, the main limiting factors which interfere with the realization of the productive potential of plum are the precipitation deficit in spring and summer against the background of intensive solar insolation and the physical and chemical properties of soil. The search of special technological methods and the selection of variety-rootstocks combinations and planting structures with compact bark that keep the plum plant resistance and the formatting of economically valuable signs of fruit quality are relevant due to the regional features of soil and climate conditions **(Sergeeva, Kuznetsova, Nenko, 2015; Sergeeva, Kuznetsova, Kovalenko, 2016)**.

Early studies have identified the nutrition mode features and the yield of *Prunus domestica* plum of varieties zoned in the region on the rootstock cherry plum seedlings. The field experiments were systematic and long-lasting which allowed us to draw a conclusion about the positive effect of regular application of mineral fertilizers on the reproductive function of the crop **(Sergeeva and Yatsenko, 1999).** Further research in this area (2014 – 2017) is linked with the intensification of production, the problem of rational selection varieties and rootstocks of *Prunus domestica* plum – components that help stabilize productivity of trees in various soil and climate area conditions for new industrial plantings **(Kuznetsova et al, 2011; Kuznetsova and Romanenko, 2014)**.

Scientific hypothesis

After analysis of dynamics of *Prunus domestica* plum plant productivity in the plantings of various structures and level of agricultural equipment in chernozem leached of the central zone of Krasnodar territory the working hypothesis about the regulation function of rootstock depending on the mineral nutrition mode of the crop that contributes to the formatting of physiological resistance to the negative impact of abiotic factors repeated in summer annually was ventured. That served as the basis for conducting experimental research in production conditions of influence of the systemic application of fertilizers on the functional state, productivity, and quality of plum fruits of the Stanley variety that is quite demanding to the soil and climate conditions on the rootstocks of various strength of growth.

MATERIAL AND METHODOLOGY

The object of research 2014 – 2017 is Prunus domestica plum plants of Stanley variety on the clonal rootstocks of various strength of growth: VVA 1, Best, PK SK 1, Pixy and cherry plum seedlings **(Figure 1)**.

Figure 1 Experienced *Prunus domestica* plum Stanley variety on the rootstocks.
Note: A – *VVA 1*, cross Microcerarus tomentosa (*Thunb) Erem. et Yushev*) x (*P.cerasifera Ehrh.*); B – *Best*, *Prunus pumila L. x Prunus cerasifera Enrh*; C – *PK SK 1*, cross of hybridization *Microcerasus pumila L. x Prunus cerasifera Ehrh*; F – *Pixy*, nursling *Prunus domestica subsp. Insititia (L.)*; G –*cherry plum seedlings (Prunus cerasifera Ehrh)*.

Rootstock characteristics: *VVA 1* is the frost-resistant, but unstable to drought rootstock of domestic selection. Plum trees come into the fruiting quickly and have a crown 50% less than ones on cherry plum seedlings.

Best is the winter-hardy resistant to high temperatures medium rootstock. It has a positive influence on the precocity and the productivity of grafted trees which have an average drought tolerance.

PK SK 1 is the frost-resistant and drought-tolerant, resistant to bacterial cancer medium rootstock of domestic selection that has high productivity of the mother plants and plum trees on it.

Pixy – the easily propagated semi-dwarf rootstock of English selection that has a positive influence on the productivity and the plum tree crown compactness and gives a lot of growth in the garden. Besides, its tolerance to drought and frost-resistance is mediocre. The disadvantages of this rootstock as all seed ones are the late entry of plum trees to fruiting, the low percentage of rootstock yield from winter with low negative temperatures, and the difficulty of use in intensive gardening.

Cherry plum seedlings – the high rootstock that is high adaptative to the wide range of stress factors affecting the South of Russia. Its compatibility with all known varieties of plum is the cause of prolonged use in the North Caucasus.

The material for the study was obtained in industrial plantings of *Prunus domestica* plum of the close corporation "Plodovod" (Krasnodar) planted in 2006. Geographically, the experimental site is located in the central plains of the Krasnodar Territory. Height above sea level varies from 19 to 32 m. The climate is temperate-continental. The layout of plants is 5 x 2 m. The trees are cultivated without any support. The system for crown formatting of plum trees is spindle-shaped ensuring the greatest productivity of the variety in thickened plantings, according to several authors **(Rakićević et al, 2007)**. The forming complex pruning was carried out in the late spring on the formed ovary of fruits. Performing corrective cropping in July allowed plants to save a full ovary and realize the generative function as much as possible. The equilibrium balanced crown with fruit buds that were formed in the last summer-early autumn and had a high winter and frost resistance was created by the subsequent removal of growth points from the vegetative shoots, the thinning, and the lightening of thickened parts. The annual spring inspection of the freezing of flower buds in trees of various growth strength showed no damage from the return of cold weather and the killing frosts after the beginning of flowering. The blooming score was 4,8-5,0 in all versions of the experiment annually.

The soil of the experimental plot is the agrosem clay-illuvial low-humus heavy-duty **(Shishov L. L et al, 2004)**. The plot is aligned according to the terrain and the value of agrochemical indicators of the soil. The agrochemical study of its soil and plants was conducted using common methods and GOST standards of chemical diagnostics **(GOST standards 26204-91; 26213-91; 26951-86; Voskresenskaya, 2006; Yaroshenko and Sergeeva, 2020)**. The complex organic-mineral chlorine-free fertilizer based on peat (Organic Mineral Fertilizer from Russian abbreviation: ОМУ) was applied at a dose of 5.0 t. ha^{-1} in the garden soil in the middles at the distance 1 – 1.2 m from the trunk of the tree in optimal agrotechnical times. The effect of Organic Mineral Fertilizer was prolonged due to the content of organic-mineral complexes with humic compounds allowing to fix nitrogen and potassium in the exchange form and decrease their mobility in the soil. The phosphorus in fertilizer was presented in a form digested easily by plants. The fertilizer was granular with a mass fraction of nitrogen (in ammonium form) of 7.0%, total phosphorus (P_2O_5) of 7.0%, potassium (K_2O) of 8.2%, humic compounds of 2.5%. The non-root dressings of 0,5% solutions of special complex mineral fertilizers were applied in combination with biologically active agents thrice during the vegetative period. The fertilizers of N18P18K18Mg1S1.5 and N12P12K35Mg2S0.7 brands were used. They included Fe, Cu, Zn, Mn, B, Mo trace elements in chelated form. The non-root cultivation with fertilizer water solutions was conducted by means of the knapsack sprayer. The version with no Organic Mineral Fertilizer application and trees spraying with no addition of any fertilizers was controlled. There were 6 accounting trees in the control and experimental version. The tests were performed in four replications. The total area of the research site – 2 ga.

Laboratory tests of plants' physiological state (determining amino acids) were conducted on three analytical surfaces according to the recommended methods **(Nenko et al, 2015)**. The chlorophyll (a+b) content in the leaves was determined in dynamics using the spectral method on the spectrophotometer Unico 2800 ("United Products & Instruments", USA). The water content in leaves was analyzed by the weight method according to the M.D. Kushnirenko technique **(Kushnirenko, 1986)**. The elemental composition plants were conducted using common methods **(Voskresenskaya, 2006)**.

The metrological observations allowed us to evaluate the degree of the negative impact of abiotic factors in the summer and establish the beginning of the stressful situation for plum plants during the study.

Agrobiology accounting was carried out according to the recommended methods **(Sedov and Ogoltsova, 1999)**.

Statistical analysis

The statistical analysis was performed in accordance with the recommended method **(Volkov, 2005)**. Calculations were performed using a software package Microsoft Office 2010 ("Microsoft, Inc.", USA). The *p*-value was used ($p < 0.05$) for checking the null hypothesis to quantify the idea of statistical significance.

RESULTS AND DISCUSSION

The agrochemical indicators of the plot soil were explored before laying the experiment. The humus content of the topsoil varied between 3.05 – 3.17% (LSD ($p \leq 0.05$) = 0.14), the mobile phosphorus one – between 243 – 252 $mg.kg^{-1}$ (LSD ($p \leq 0.05$) = 7.70), the exchangeable potassium content – between 217 – 221 mg. kg^{-1} (LSD ($p \leq 005$) = 3.42). The content of the nitrate form of nitrogen in the soil layer 0-60 sm did not exceed 0.7 – 1.2 mg. kg^{-1} The chemical analysis showed a significant increase of the mineral elements forms available for plants one year after the application of mineral fertilizers. The mobile phosphorous content was 286 – 308 mg. kg^{-1}, the exchangeable potassium one was 238 – 245 mg. kg^{-1} in the mineral fertilizer application zone.

Table 1 Effect of fertilizers on the content of macronutrients in the leaves of plum, the third decade of may (mg.kg^{-1}).

Variation	N	P	K	Ca	Mg
			Rootstock VVA 1		
Control	3.08	0.34	1.72	1.01	0.35
OMF+ foliar application	3.56	0.35	1.76	1.18	0.43
LSD ($p \leq 0.05$)	0.24	0.05	0.07	0.13	0.06
			Rootstock Best		
Control	3.16	0.33	1.54	1.26	0.58
OMF+ foliar application	3.75	0.36	1.65	1.48	0.69
LSD ($p \leq 0.05$)	0.10	0.03	0.07	0.11	0.09
			Rootstock PK SK 1		
Control	3.11	0.33	1.69	1.18	0.61
OMF+ foliar application	3.45	0.35	1.72	1.23	0.64
LSD ($p \leq 0.05$)	0.15	0.04	0.03	0.17	0.08
			Rootstock Pixy		
Control	3.19	0.33	1.66	1.51	0.44
OMF+ foliar application	3.61	0.36	1.73	1.49	0.43
LSD ($p \leq 0.05$)	0.13	0.02	0.05	0.05	0.02
			Rootstock cherry plum seedlings		
Control	3.42	0.34	1.97	1.68	0.50
OMF+ foliar application	3.69	0.35	1.95	1.71	0.56
LSD ($p \leq 0.05$)	0.14	0.03	0.07	0.04	0.07

The nitrate-nitrogen content in the soil layer 0 – 40 cm increased 3 – 5 times in spring and 97 – 155% in October compared to the control version (without Organic Mineral Fertilizer application). Against that background the first non-root dressing of trees with water solutions of special fertilizers was conducted in the ratio N:Ph:P = 1:1:1. The treatment was repeated 4 – 5 weeks after flowering (the fruit size is up to 3 cm in diameter) during the stone formatting. The third dressing with water solutions of special fertilizers was conducted in the ratio N:Ph:P = 1:1:2 – 2.5 in summer (in the period of differentiation of buds). The phytoregulator "Novosil" was added in the nutrient solution composition in the ratio at the concentration of 0.02% at each treatment of plants. The changes in the seasonal nutrition mode were checked for the nutrients content in the leaves of growth shoots **(Table 1)**.

Annual systematic application of fertilizers by non-root method during the period of the intensive shoot and ovary growth (in spring) promoted increasing of the total content of nutrients in plum leaves on the rootstocks of various strength of growth. The resulting effect was also noted by several authors who have studied the influence of fertilizers on the plum nutrition mode (Miletić, et al, 2003; Fatma et al, 2018).

The significant increase was detected mainly in the nitrogen and potassium content. However, the increase in potassium content was not always confirmed statistically. Besides, the reliable increase of potassium and magnesium content was noted in the plum on the *VVA 1* dwarf rootstock during that period.

The changes in the plum nutrition mode promoted activation of physiological processes. This corresponds to the data from literature sources reporting about the positive impact of fertilizers on the stone fruit crops **(Wan Yi-zhen, Sheng Bo-biao, Du Hui-fang, 2003; Miletic et al, 2003; Cuque et al, 2011; Milosevic and Milocevic, 2012; Doroshenko, Riazanova, Maksimtsov, 2015; Fatma et al, 2018)**. The increase of several functional pigments (Chla+Chlb) in the plum leaves on rootstocks of various strength of growth was identified by the spectral analysis method in the application of fertilizers. The most significant increase of a number of the main photosynthetic pigment (Chla), an energy donor, was discovered in the leaves of plum on the rootstock *Best.* The high content of the chlorophyll b (Chlb), the activator of the biosynthetic processes, was occurred in the same version annually. The total chlorophyll content (Chla+Chlb) in the leaves exceeded stably the value in the control version by 20.1-25.4% in trees on the rootstock *PK SK 1, rootstock Pixy,* and rootstock *cherry plum seedlings*. At the same time, the lamina area of trees on those rootstocks exceeded the values in the control version by 12.7%, 7.4, and 9.1% respectively. The tendency to increase the nutrient content in the plum leaves against the background of the application of fertilizers revealed mainly in the years with the sufficient water availability on the *VVA 1* dwarf rootstock that had the

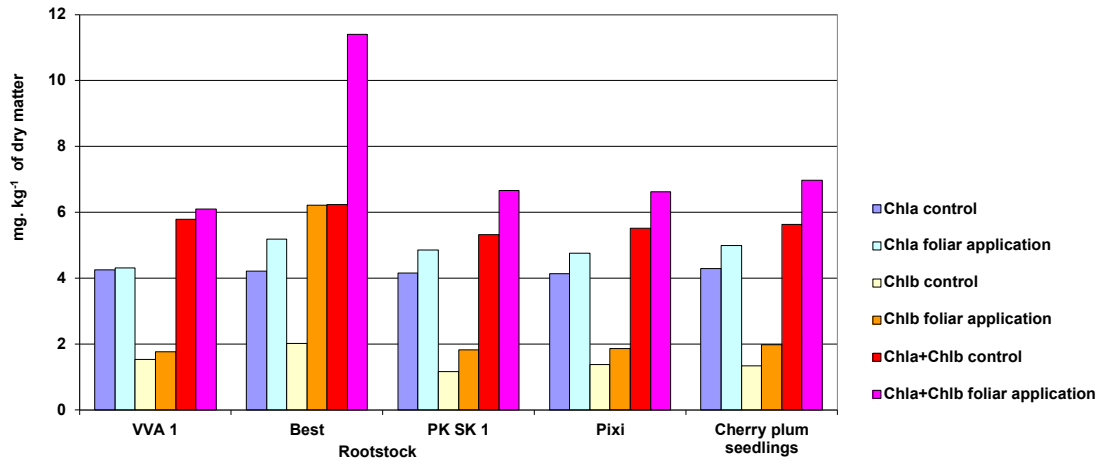

Figure 2 The content of functional pigment in plum leaves in relation to the fertilizers and variety-rootstock. combinations.

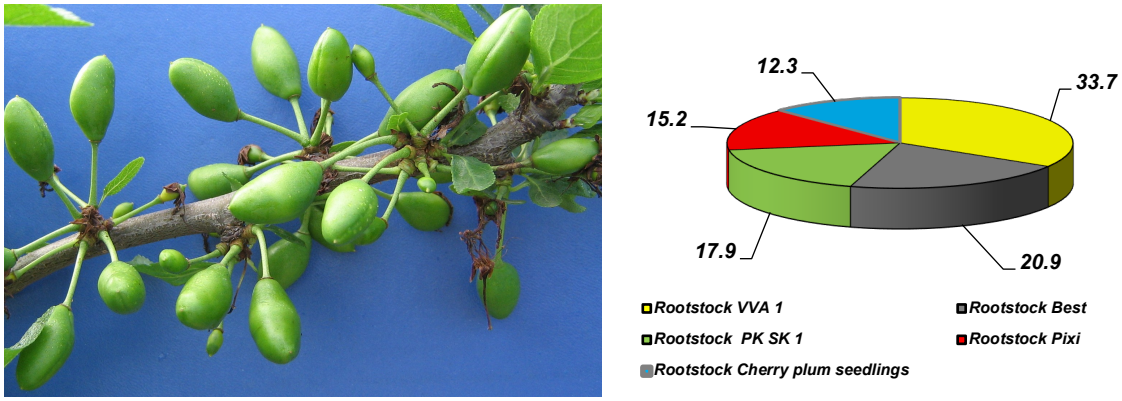

Figure 3 The excess of the number of preserved ovaries in plum plants against the background of application of fertilizers in relation to the control version (with no fertilizers) (the third decade of June),% (2014 – 2017).

surface occurrence of the main part of the root system **(Figure 2)**.

The increase of the intensity of metabolic processes that was probably associated with the activity of photosynthesis and synthetic activity of the root system was confirmed by the analysis of the free amino acid content in the leaves using the method of capillary electrophoresis.

The tendency of the predominant increase of arginine in shoot leaves was detected annually during the period of the activity of growth processes in spring when the maximum temperature was up to 28 – 32 ºC and the precipitation fell periodically. The changes in the nutrition mode promoted increasing of the arginine amount in leaves by 42.3% (*Rootstock Best*), 37.4% (*Rootstock PK SK 1*), 29.6% (*Rootstock Pixi*), 12.7% (*Rootstock VVA 1*), and 27.9% (*Rootstock cherry plum seedlings*) at that stage of seasonal plant development. More active protein synthesis was occurred in leaves on the rootstock *Best* in spring and summer annually. Its content was higher by 8-10% compared to the one in the control version (with no fertilizers). Further, the recorded annual increase in air temperature to maximum values of 30 – 35 ºC and the prolonged lack of precipitation promoted increasing of the amino acid Proline content in the leaves. Its content in leaves was 46.4% (*Rootstock PK SK 1*), 39.8% *(Rootstock Pixi*), 32.5% (*Rootstock VVA 1*), 26.2% (*Rootstock cherry plum seedlings*), and more than 80% (*Rootstock Best*) higher in the version with the application of fertilizers. The identified tendency was confirmed by our researches on other fruit crops **(Popova, Yaroshenko, Sergeeva, 2018)**.

The nutrition mode of culture changed by the end of the second decade of June against the background of the growth activity decrease of trees. The total nitrogen and phosphorous content in all versions of experiment was 2.22 – 2.24 and 0.163 – 0.168% respectively. The potassium content went up to 2.12% (*Rootstock VVA 1*) and 2.61 – 2.68% (*Rootstock Best, Rootstock PK SK1, Rootstock Pixi, Rootstock cherry plum seedlings*). Calcium in the leaves of dwarf and medium trees went up to 1.59% (*Rootstock VVA 1*) and 1.70% respectively. There were no changes in the plum on the rootstock *cherry plum seedlings*. The total magnesium content changed insignificantly. The secondary metabolite content in the plum shoot leaves decreased on the rootstocks of various strength of growth. That was probably caused by an outflow of assimilates into the growing fruits. The total number of amino acids was no more than 60.2 -89.6% of the previously established content in all versions of the experiment. In June the accountings for the degree of ovary reduction on the accounting trees showed the advantage of the version with the application of fertilizers: a number of the fully preserved ovary was higher by 12.3 – 33.7% depending on the rootstock **(Figure 3)**.

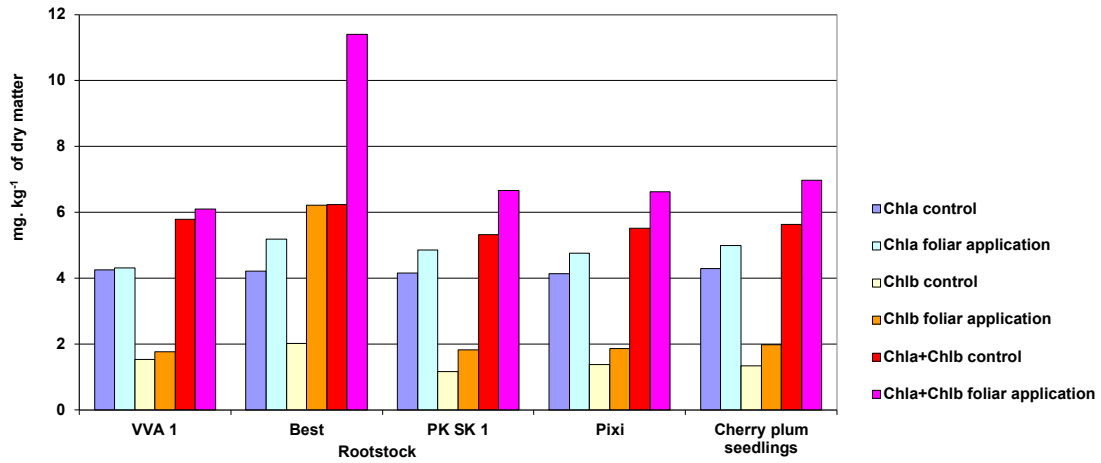

Figure 4 The most common character deformity of plum ovary development on the example of the Stanley variety on the rootstock *Best* (2014 – 2017).

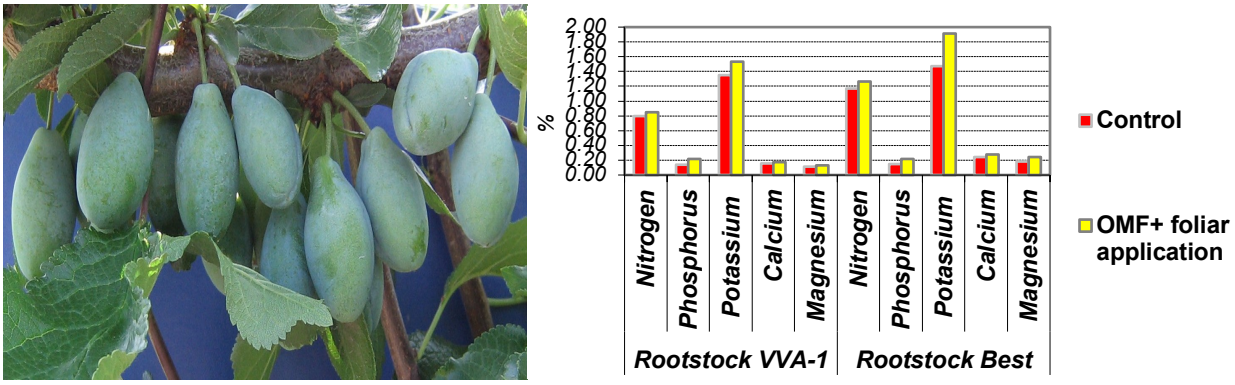

Figure 5 The chemical content of growing fruits in July (2014 – 2017).

The visual analysis and the calculation of the ovary in the control version allowed us to conclude that without the application of fertilizers, the percentage of deformed fruits, that is associated, according to **Semenov (2007)**, with the negative abiotic factors impact, is higher significantly and ranges from 3.3 to 8.7% depending on the variety-rootstock combination. The most common characteristics of ovary deformity are represented in **Figure 4**.

In July the stage of development of generative buds of plum of Stanley variety in the sinuses of side leaves of shoots was diagnosed by the method of microscopy. In the same period, the analysis of the chemical content of growing fruits was conducted with leaf diagnostics. The most significant difference between the versions was identified on the rootstock *VVA*1 and rootstock *Best* **(Figure 5)**. The closest correlation between the nitrogen, potassium, and calcium content in leaves and fruits was determined. The correlation coefficient was 0.68, 0.74, 0.52 (*Rootstock VVA 1*) and 0.75, 0.84, 0.61 (*Rootstock Best*) respectively.

The correlation analysis showed a less close relationship that did not exceed the values of 0.55 – 0.64. Several scientists **(Plich, Wójcik, 2002; Fatma et al, 2018)** pointed to changes in the quality of plum fruits and the increase of calcium content in them against the background of the application of non-root dressings.

The summer and early autumn experimental studies were accompanied by the annual accounting and the analysis of metadata because many authors pointed to the strong influence of the water stress factor on the intensity of plum growth processes **(Li Wen-hua et al, 2003)**. It is necessary to note that the longest stress situations related to hyperthermia, the lack of precipitation, and the decrease of air humidity up to 12 – 14% was observed from the second decade of July to mid-September. The data of the functional state of Prunus domestica plum plants on the rootstocks of various strength of growth was the most informative and allowing to evaluate the effectiveness of fertilizers under these conditions.

Conducted research on green pigment content in leaves identified the seasonal decrease of plant photosynthetic activity. At the same time, despite the negative effect of physical factors the level of green pigment content in plum shoot leaves was higher significantly against the background of the application of fertilizers than one in the control version. It is possibly related to the action of the phytoregulator used as a part of the nutrient solution for non-root dressings and increased the plant protective features. That fact was also pointed by in their study **(Upadysheva, Upadyshev, 2012) (Table 2)**. The difference between the experimental versions was also identified by protein content in plum leaves. The seasonal decrease of protein in shoot leaves an average of 20-24% compared to the spring and the summer period was observed in all versions of the experiment. However, when the fertilizers were applied the decrease of protein amount in leaves of plum on the rootstock *Best* and rootstock *PK SK 1* was minimal and amounted to 25.3 and 23.2 mg. kg^{-1} of dry matter respectively.

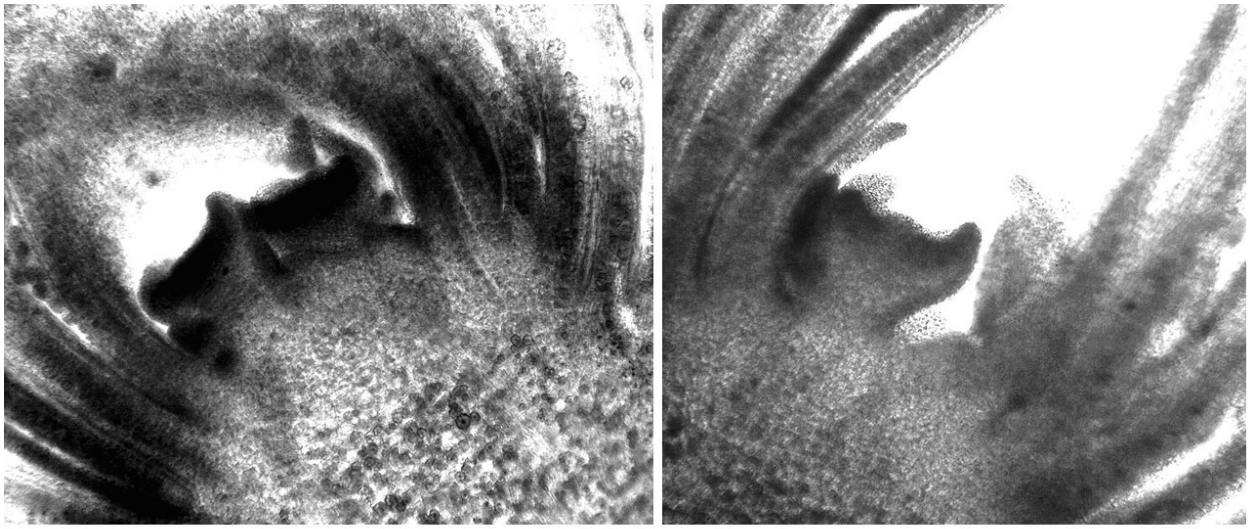

Figure 6 The development of the sepal rudiments in the inflorescence flower (A), the development of the sepal rudiments in the single flower (B) (2014 – 2017).

Table 3 Effect of fertilizers on the content of macronutrients in the leaves of *Prunus domestica* plum, early August ($mg.kg^{-1}$).

Variation	N	P	K	Ca	Mg
			Rootstock VVA 1		
Control	2.41	0.18	1.87	2.73	0.37
OMF+ foliar application	2.62	0.18	2.80	3.17	0.51
LSD ($p \leq 0.05$)	0.11	0.02	0.18	0.14	0.10
			Rootstock Best		
Control	2.52	0.14	2.58	3.19	0.34
OMF+ foliar application	2.58	0.16	2.75	3.48	0.46
LSD ($p \leq 0.05$)	0.07	0.02	0.08	0.11	0.05
			Rootstock PK SK 1		
Control	2.64	0.16	2.32	3.12	0.38
OMF+ foliar application	2.86	0.16	2.44	3.17	0.41
LSD ($p \leq 0.05$)	0.09	0.01	0.07	0.10	0.05
			Rootstock Pixy		
Control	2.47	0.14	2.49	3.03	0.43
OMF+ foliar application	2.53	0.15	2.57	3.10	0.48
LSD ($p \leq 0.05$)	0.05	0.02	0.05	0.09	0.05
			Rootstock cherry plum seedlings		
Control	2.73	0.20	2.48	1.85	0.34
OMF+ foliar application	3.03	0.23	2.97	2.97	0.35
LSD ($p \leq 0.05$)	0.13	0.02	0.19	0.23	0.06

That fact characterized higher intensity of reparation processes in trees on those rootstocks.

Under the conditions of the tensity of hydrothermal factors in summer, the water content in leaves was determined depending on the application of fertilizers and the variety-rootstock combinations. Plants on the rootstock cherry plum seedlings had the most significant losses of shared water that was up to 9.3% (Organic Mineral Fertilizer + non-root dressings) and 12.7% (control, with no fertilizers). The minimal water losses were identified in leaves in plum on the rootstock *PK SK 1* that was 5.7 – 8.4% respectively.

The continuation of diagnostic studies of the functional state of the plum plant was accompanied by morphoanatomical observations of the generative buds in the first decade of August. The beginning of the differentiation of the rudimental sepals was detected in the majority of conducted buds **(Figure 6)**.

That stage of plum plant development was characterized by the next change of nutrition mode. The gross content of nitrogen, potassium, and calcium increased in shoot leaves. At the same time, the differences between the versions were significant **(Table 3)**.

According to the morphoanatomical research data, the transition to formatting of petals and stamens began in the majority of rudimental flowers of the plum plant by early September. The periodical precipitation promoted growth activity mainly in trees on the medium rootstocks. The gross content of nitrogen, phosphorous, and magnesium increased up to 18.6%, 7.7, and 29.5% respectively regardless of the version of the experiment **(Figure 7)**.

The most significant increase of amino acid content in leaves was determined in plum plants on the rootstock *Best* in the version with the application of fertilizers against that background. The excess of the sum of amino acids was 26.6 – 30.8% in some years. The correlation coefficient between the nitrogen content in leaves and the total amount of free amino acids was r = 0.56 (Rootstock *VVA 1*), r = 0.67 – 0.71 (Rootstock *PK SK 1*, Rootstock *cherry plum seedlings*), r = 0.81 – 0.83 (Rootstock *Best*).

The specified period was also the beginning of fruit ripening of plum of Stanley variety **(Figure 8),** which had an immediate influence on the post-harvest storage of fruits, according to the authors **(Manganaris, Vicente, Crisosto, 2008)**.

The significant impact of the fertilizers applying in the experiment on the content of gross forms of potassium and calcium in fruits on the rootstock *VVA 1*, rootstock *Best*, rootstock *PK SK 1* those content exceeded the amount of the element in fruits of the control version by 12.5 and 23.5%; 24.2 and 11.5%; 17.3 and 19.6% respectively.

The dynamics of plum productivity on the rootstocks of various strength of growth against the background of the application of fertilizers were analyzed depending on the protection area of tree crowns **(Table 4)**. The dimensions of plum tree crowns formed by a spindle-shaped system matched the strength of the growth of the rootstocks. The annual measurement of the protection area of tree crowns in 2014 – 2018 allowed us to establish the highest values in trees on the rootstock *cherry plum seedlings* that were amounted to 3.69 – 3.99 m^2. The smallest protection area of tree crowns was noted in the trees on the rootstock *VVA 1*

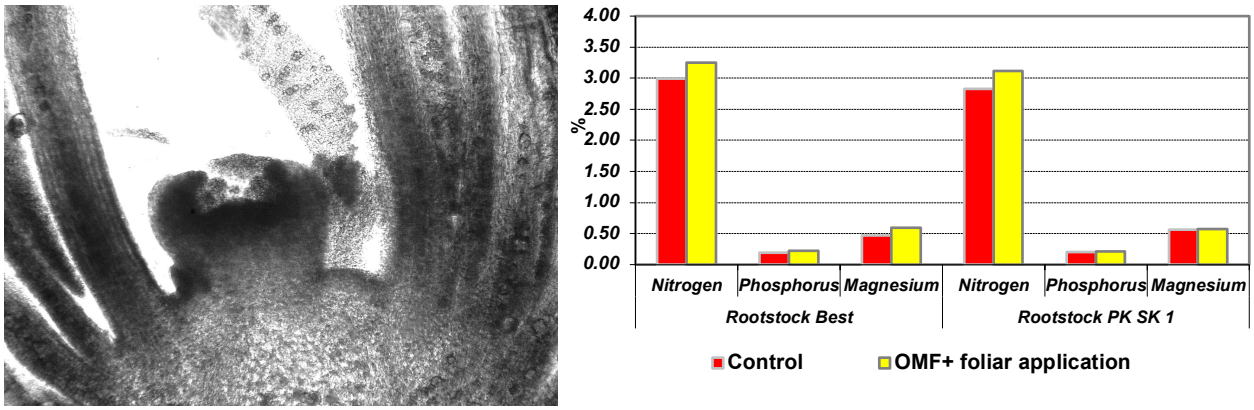

Figure 7 The nitrogen, phosphorous and magnesium content in shoot leaves of plum of Slanley variety in the stage of forming of the petals and stamens in the rudimental flowers, August-September (2014 – 2017).

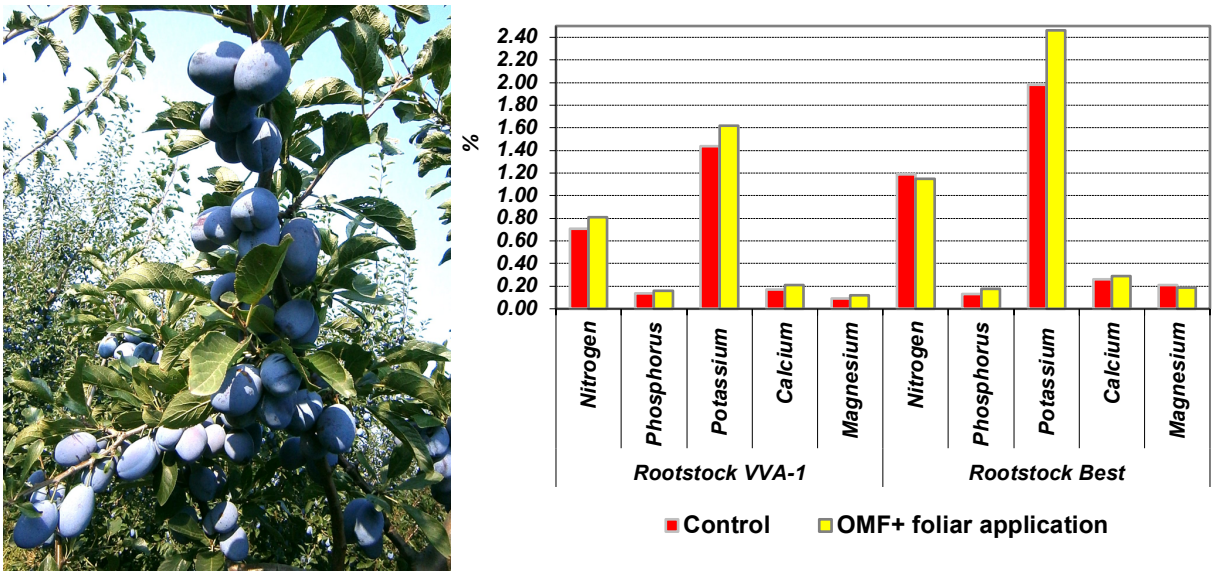

Figure 8 The chemical content of fruits in the ripening period, August-September (2014 – 2017).

Table 4 The productivity of plum of Stanley variety depending on the habitus of the crown and the application of fertilizers.

The number of fruits per 1 m^2 of the crown projection area, piece.				
Variation	2014	2015	2016	2017
		Rootstock VVA 1		
Control	245	237	288	307
OMF+ foliar application	334	354	345	352
LSD ($p \leq 0.05$)	21.42	20.48	21.87	11.78
		Rootstock Best		
Control	210	259	295	286
OMF+ foliar application	240	267	338	321
LSD ($p \leq 0.05$)	8.22	5.44	16.98	7.52
		Rootstock PK SK 1		
Control	173	212	225	200
OMF+ foliar application	197	255	241	236
LSD ($p \leq 0.05$)	22.41	42.47	13.39	27.28
		Rootstock Pixy		
Control	192	229	237	256
OMF+ foliar application	231	261	258	278
LSD ($p \leq 0.05$)	25.01	20.68	24.08	25.76
		Rootstock cherry plum seedlings		
Control	257	248	261	254
OMF+ foliar application	266	274	279	283
LSD ($p \leq 0.05$)	22.81	12.04	15.63	7.28

that was 2.02 – 2.52 m^2. The average indicators were determined on the rootstock *Best*, rootstock *PK SK 1*, and rootstock *Pixi* that are 3.07-3.13; 3.53-3.75 and 3.36-3.64 m^2 respectively. The highest stable indicators of plum productivity were identified on the dwarf rootstock *VVA 1* that matched the data of **(Provorchenko et al, 2015)**.

The applied fertilizer system promoted significant excess of reproductive function of plants. Meanwhile, the slopes of trees on the rootstock *VVA 1* caused by harvest load were observed. That indicated the need for installation of supports by using the rootstock *VVA 1*. At the same time, that crown design and the dimensions of the trees allowed us to decrease significantly the plant nutrition area without a decline of the light regime that is also pointed by **Upadysheva, 2015** in her research. The productivity of plum on the medium rootstocks was less significant, but the advantage of the version with the application of fertilizers was saved. The most significant and stable indicators of growth of productivity were received in the versions on the rootstock *Best* (*LSD* ($p \leq 0.05$) – 8.22 (2014); 5.44 (2015); 16.98 (2016); 7.52 (2017)) against the background of the application of fertilizers in those soil and climate conditions. At the same time, it is necessary to note that the values of indicators in the limits of the version were changeable significantly in trees on the rootstock *PK SK 1*, rootstock *Pixi* and rootstock *cherry plum seedlings.*

The classification of plum fruits by quality was conducted according to GOST standard 21920-76 in all versions of the experiment. Such indicators as the color of the fruit, the appearance, the presence of the damages on the surface, the turgescence, and the homogeneity were identified on the level of standard that is common for the feature of plum of European varieties **(Ionica et al, 2013).** The significant differences in the dry matter content in fruits on the rootstocks of various strength of growth were not determined that was accorded with the data of the next scientists **(Meland and Froynes, 2006)**. The sort (calibration) of fruits allowed us to identify the advantages of the version with the application of fertilizers by the criterion "the weight of fruit". The optimization of the nutrition mode of plum of Stanley variety promoted a significant increase in the fruit weight no depending on the strength of growth of the rootstock. That fact matched the data of literature sources **(Kaufmane et al, 2007, Vetrova and Roeva, 2019) (Table 5)**.

Table 5 The weight of plum fruit of Stanley variety depending on the application of fertilizers.

	The mass of one fruit (g).			
Variation	2014	2015	2016	2017
		Rootstock VVA 1		
Control	41.7	40.9	34.8	38.8
OMF+ foliar application	42.5	42.7	36.7	40.6
LSD ($p \leq 0.05$)	0.59	1.05	1.34	0.97
		Rootstock Best		
Control	40.3	41.1	32.6	35.7
OMF+ foliar application	40.8	41.9	33.6	37.8
LSD ($p \leq 0.05$)	0.56	0.71	1.02	1.35
		Rootstock PK SK 1		
Control	39.2	40.1	37.5	40.1
OMF+ foliar application	40.6	41.8	37.9	40.7
LSD ($p \leq 0.05$)	1.04	0.43	1.42	0.78
		Rootstock Pixy		
Control	38.8	37.2	35.5	38.9
OMF+ foliar application	39.4	40.5	36.7	39.6
LSD ($p \leq 0.05$)	0.63	0.77	0.68	0.80
		Rootstock cherry plum seedlings		
Control	37.9	39.6	33.2	38.1
OMF+ foliar application	38.6	40.3	33.3	38.7
LSD ($p \leq 0.05$)	0.37	0.56	0.38	0.77

CONCLUSION

The impact of the rootstock and the systematic application of intra-soil and leaf dressings in combination with the phytoregulator on the physiological resistance of plum plants of Stanley variety to the negative effect of annually repeated abiotic factors in summer was investigated according to the criterion of functional state, productivity, and quality of fruits.

As a result of the analysis of experimental data, we identified that the application of fertilizers promoted a significant increase of total content of biologically essential elements and enhanced the synthesis of functional pigments (Chla+Chlb) in spring during the period of the intensive shoot and ovary growth in the plum tree on rootstocks of various strength of growth. The total chlorophyll content (Chla+Chlb) in the leaves exceeded stably the value in the control version by 20.1 – 25.4% in trees on the rootstock *PK SK 1*, rootstock *Pixy*, rootstock *cherry plum seedlings*. The effectiveness of the influence of the nutrition mode revealed mostly in the years with the sufficient water availability on the dwarf rootstock *VVA 1* that has the surface occurrence of the main part of the root system. The increase of the growth process activity was confirmed by the increase of the content of free amino acids that were the most active participants in metabolism in the leaves.

The conditions of mineral nutrition mode influenced the chlorophyll synthesis in plum leaves on the rootstock *VVA 1*, rootstock *Best*, rootstock *PK SK 1*and rootstock *cherry plum seedlings* in summer against the background of hyperthermia, the lack of precipitation, and the decrease of air humidity up to 12 – 14%. The specific to these conditions decline of protein content in the trees on the rootstock *Best* and rootstock *PK SK 1* was minimal. That fact characterized higher intensity of reparation processes. Plants on the rootstock *cherry plum seedlings* had the most significant losses of shared water that were up to 9.3% (Organic Mineral Fertilizer + non-root dressings) and 12.7% (control, with no fertilizers). The minimal water losses were identified in leaves in plum on the rootstock *PK SK 1* that was 5.7 – 8.4% respectively.

The periodical precipitation in early September promoted growth activity mainly in trees on the medium rootstocks. The period of growth activity was accompanied by the increase of gross content of nitrogen, phosphorous, and magnesium that was up to 18.6%, 7.7, and 29.5% respectively. The most significant increase of amino acid

content in leaves was determined in plum plants on the rootstock *Best* against the background of the application of fertilizers. The excess of the sum of amino acids was 26.6 – 30.8% in some years.

The period of the beginning of fruit ripening was characterized by the accumulation of gross forms of potassium and calcium in them. The potassium and calcium content in fruits in the version with the application of fertilizers exceeded the amount of the elements in fruits of control version by 12.5 and 23.5%; 24.2 and 11.5%; 17.3 and 19.6% on the rootstock *VVA 1*, rootstock *Best*, rootstock *PK SK 1* respectively. At the same time, the harvest accountings revealed the advantage of the versions with the application of fertilizers: there was an increase in productivity due to a decline of ovary reduction in spring and summer. The number of the fully preserved ovary was higher by 12.3-33.7% depending on the rootstock.

The commercial quality analysis by the criterion "the weight of fruit" that identified the level of economic feasibility of the adoption of optimization of culture's mineral nutrition mode showed the advantages of the version with the application of fertilizers. The largest fruits were obtained from the trees on the rootstock *VVA 1* and rootstock *Best.*

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ESSENTIAL OILS AND THEIR APPLICATION IN A FOOD MODEL

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ABSTRACT

The aim of the study was to investigate the chemical composition, antioxidant, and antimicrobial activity of essential oils (*Canarium luzonicum* CLEO, *Melaleuca leucadenron* MLEO, *Amyris balsamifera* ABEO). There was Gas chromatographic-mass spectrometric analysis used for the characteristic of the semiquantitative composition of the essential oils. The DPPH method was used to determine the antioxidant activity. Minimum inhibitory concentrations (MIC) of essential oils against *Stenotrophomonas maltophilia* were analyzed in a 96-well plate. The broth microdilution method was used for the minimal inhibitory concentration. A gas-phase antimicrobial assay was used to determine inhibitory concentrations in a food model. CLEO proved to be the best with the lowest MIC 50 and 90 of 6.67 µL.mL^{-1} respectively 6.81 µL.mL^{-1} and antioxidant activity of 33.43% among the tested essential oils. The main volatile compounds CLEO were limonene 36.38%, elemol 16.65%, α-fellandren 12.18% and elemicin 9.59%. It showed inhibition of *S. maltophilia* growth in the food model at the lowest concentrations among the essential oils.

Keywords: *Stenotrophomonas maltophilia*; *Canarium luzonicum*; *Melaleuca leucadenron*; *Amyris balsamifera*; essential oil; food model

INTRODUCTION

In recent years, natural substances have come to the fore due to their low toxicity, pharmacological effects, and economic advantage **(Dias, Urban, and Roessner, 2012)**.

Elemi (*Canarium luzonicum*) essential oil comes from an evergreen tree that reaches a height of more than 30 meters and a trunk diameter of more than 1 meter. *C. luzonicum* naturally occurs in the Philippines **(Barwick, Schans and Claudy, 2004)**.

Oleoresin is one of the aromatic components. Therefore, it has a wide range of uses in the pharmaceutical and food industries. It is also used for its rubefic, expectorant, antifungal, antibacterial and antirheumatic effects **(Nikolic et al., 2016)**.

Kajeput essential oil (*Melaleuca leucadendron*) is used for its antifungal, antiviral, antibacterial, antiseptic, and anti-inflammatory effects. The plant occurs predominantly in Indonesia **(Pujiarti, Ohtani and Ichiura, 2011)**. Many of the compounds present in this plant are considered to be bioactive substances **(Cleber et al., 2007)**.

Amyris, essential oil (*Amyris balsamifera*) comes from always green small trees and it has high flammability. It occurs in the Caribbean and near the Gulf of Mexico **(Rohmer, Schwartz and Anton, 2012)**. Amyris is rich in sesquiterpene alcohol. It has antiseptic effects **(Khan and Abourashed, 2009)**.

Stenotrophomonas maltophilia is a non-fermentative, gram-negative, aerobic bacillus. These bacteria can form biofilm structures. It is most often found in raw milk, vegetables, fruits and fish products regarding the food industry **(An and Berg, 2018)**.

We aimed to determine chemical composition, antioxidant activity, and minimal inhibitory concentrations of these essential oils against the bacterium *Stenotrophomonas maltophilia.*

Another aim was also to evaluate the inhibitory effect of essential oils against *S. maltophilia* from the surface of carrots, potatoes and apples by using a vapor phase antimicrobial test.

Scientific hypothesis

We assume the presence of biologically active substances and the antioxidant potential of essential oils. Given the available literature, we assume the inhibitory effect of essential oils on the bacteria *Stenotrophomonas maltophilia*. We believe that essential oils could also have an inhibitory effect in the vapor phase.

MATERIAL AND METHODOLOGY

Essential oil

The tested essential oils (*Canarium luzonicum* CLEO, *Melaleuca leucadenron* MLEO, *Amyris balsamifera* ABEO) were bought from the Hanus s.r.o (Slovakia).

Microorganism

Bacteria *Stenotrophomonas maltophilia* was got from the dairy industry. It was identified by 16S rRNA sequencing and MALDI-TOF MS Biotyper.

Chemical Composition of Essential Oils

There was Gas chromatographic-mass spectrometric (GC-MS, Agilent 7890B, Agilent 5977A, Agilent Technologies Inc., Palo Alto, CA, USA) and CombiPal autosampler 120 (CTC Analytics AG, Zwingen, Switzerland) parsing test of the essential oil used as well as in a previous study **(Kačániová et al., 2020)**. The results were set as the average mean and standard deviation of three repeated measurements.

Radical Scavenging Activity—DPPH Method

The activity of capturing free radicals with essential oil was determined in the same way as in the study of **Kačániová et al. (2020)** by using 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, Germany) method.

Minimum Inhibitory Concentration (MIC)

The bacterial culture was cultivated in the Muller Hinton broth (MHB, Oxoid, Basingstoke, UK)) at 37 °C for 24 h. One hundred μL inoculum with a density of 0.5 McF was inoculated into each well of a 96-well microtitration plate. There was 100 μl of the essential oil with a concentration from 0.3125 μL to 10 μL per well added after inoculation. Negative control contended a mixture of MHB with essential oil, while a mix of MHB with bacterial inoculum was used as a control of maximum growth **(Hassan et al. 2011)**. The absorbance was measured and evaluated in the same way as it was in the study of **Kačániová et al. (2020)**. The experiment was carried out in three repeated measurements.

Statistical analysis

The measurements were repeated three times. Statistical variability of the data was processed with Microsoft™ Excel® software.

Table 1 Main components of essential oil *Canarium luzonicum.*

Name	Synonyms	TIC% Area [a]
β-phellandrene		4.54 ±0.04
α-phellandrene		12.2 ±0.05
β-pinen		0.61 ±0.03
α-terpinene		0.61 ±0.01
d-limonene		36.4 ±0.16
cis-sabinene		3.06 ±0.03
α-ocimene		0.38 ±0.01
γ-terpinen		0.58 ±0.02
cymene		3.35 ±0.01
α-terpinolen		1.59 ±0.02
4,8,8-trimethyl-2-methylene-4-vinylbicyclo[5.2.0]nonane		0.51 ±0.02
terpinen-4-ol		1.15 ±0.01
α-terpineol		3.83 ±0.01
α-phellandrene epoxide		0.39 ±0.03
1,3,4-eugenol methyl ether	4-allylveratrole	0.69 ±0.01
elemol		16.7 ±0.18
guaiol		0.43 ±0.02
10-epi-γ-eudesmol		1.59 ±0.01
γ-eudesmol		0.84 ±0.01
rosifoliol		1.08 ±0.01
elemicin		9.59 ±0.11

Note: [a] mean value ±SE.

Results The MIC value (concentration causing 50% and 90% reduction in bacteria growth) was determined by logit analysis. Statistical evaluation of the antioxidant activity of the obtained data was performed using the GraphPad Prism 8.0.1 (GraphPad Software Incorporated, San Diego, California, USA). One way analysis of variance (ANOVA) followed by the Tukey test was used for statistical analysis.

RESULTS AND DISCUSSION

Chemical Composition of Essential Oils

The main volatile compounds of the analyzed essential oil CLEO based on reduced percentage were limonene 36.38%, elemol 16.65%, α-fellandren 12.18% and elemicin 9.59% **(Table 1)**. **Swift (2002)** has stated 59.4%, α-phelandrene 8.01%, and sabinene 3.35% as the main constituents of *Canarium luzonicum*.

Orchard et al. (2017) found out that the main components of the essential oil *C. luzonicum* were limonene 47.5%, elemol 18.4%, and α-phelandrene 9.2%.

Malik (2019) indicated sabinene 5.7%, α-phelandrene 17.6%, limonene 56%, and elemol 6.3% as the main components of the essential oil *C. luzonicum*. **Silva et al. (2012)** identified limonene, β-Cymene, β-fellandren, α-phellandren and β-pinene as the main components of C. luzonicum essential oil.

The main volatile compounds of the analyzed MLEO based on the reduced percentage were eucalyptol 49.23%, α-terpineol 9.92%, limonene 8.12%, and caryophyllene 5.65% **(Table 2)**. **Pujiarti et al. (2011)** in their study tested 9 varieties of *M. leucadendron* from Java and Indonesia, in which twenty-six compounds were identified.

Table 2 Main components of essential oil *Melaleuca leucadenron.*

Name	Synonyms	TIC% Area [a]
3-carene		0.25 ±0.02
α-phellandrene		0.26 ±0.03
β-pipene		0.83 ±0.01
α-terpinene		0.48 ±0.01
d-limonene		8.12 ±0.04
eucalyptol	1,8-epoxy-p-menthane; 1,8-cineol	49.2 ±0.18
γ-terpinene		2.91 ±0.01
4-cymene		3.16 ±0.01
α-terpinolen		1.24 ±0.01
α-copaen		0.29 ±0.01
linalyl butanoate	linalyl butyrate	1.13 ±0.01
caryophyllene		5.65 ±0.03
p-menth-1-en-4-ol	1-terpinen-4-ol	0.83 ±0.03
2,4-dihydroxy-2-methylpentane	hexylene glycol	4.11 ±0.02
1,5,9,9-tetramethyl-1,4,7-cycloundecatriene -,		2.91 ±0.01
β-maaliene		0.43 ±0.01
α-muurolene		0.53 ±0.01
β-cadinene		0.65 ±0.01
α-terpineol acetate		1.84 ±0.01
α-terpineol		9.92 ±0.04
α-selinene		2.09 ±0.01
eudesma-3,7(11)-diene	selina-3,7(11)-diene	0.39 ±0.02
bicyclogermacrene	lepidozene; isolepidozene	0.36 ±0.03
caryophylene oxide		0.31 ±0.02
globulol	ledol	1.09 ±0.01
1,2-diacetate-1,2,3-propanetriol	1,2-diacetin	0.26 ±0.02

Note: [a] mean value ±SE.

Table 3 Main components of essential oil *Amyris balsamifera.*

Name	Synonyms	TIC% Area [a]
amorpha-4,11-diene	muurola-4,11-diene	2.58 ±0.02
β-cadinene		0.73 ±0.01
β-chamigrene		0.36 ±0.01
4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene		0.29 ±0.02
δ-bisabolene		0.95 ±0.01
α-zingiberene		2.21 ±0.01
β-bisabolene		0.97 ±0.01
α-maaliene		0.56 ±0.03
β-maaliene		0.32 ±0.02
cedrene	β-funebrene	4.91 ±0.02
α-curcumen		2.44 ±0.01
nerolidol		1.57 ±0.03
α-chamigrene		0.56 ±0.04
elemol		9.62 ±0.01
10-epi-γ-eudesmol		14.7 ±0.01
β-eudesmol		0.78 ±0.02
γ-eudesmol	machilol; selinenol	2.49 ±0.04
β-cadinene		0.60 ±0.01
β-guaiene	azulene	0.46 ±0.01
8-epi-γ-eudesmol		0.40 ±0.03
valerianol		23.2 ±0.16
guaiol		19.4 ±0.16
1,2,3,6-tetramethylbicyclo[2.2.2]octa-2,5-diene		0.53 ±0.02
bisabolone		0.99 ±0.01
selin-6-en-4α-ol	eudesm-6-en-4α-ol	0.27 ±0.03
β-vetispirene	β-vatirenene; β -vetivenene	0.55 ±0.03
isolongifolol, methyl ether		0.94 ±0.01
2-phenylethyl iodide		0.90 ±0.02
7-epi-γ-eudesmol		0.35 ±0.02
drim-7-en-11-ol		1.84 ±0.01

Note: [a] mean value ±SE.

These samples had a very similar composition. The results showed that 1,8-cineole (eucalyptol; 44.76 – 60.19%) was the main compound in these oils, followed by α-terpineol (5.93 – 12.45%), limonene (4.45 – 8.85%) and β-carophyllene (3.78 – 7.64%). **Sharifi-Rad et al. (2017)** reported *M. leucadendron* terpinen-4-ol 30%, 1,8-cyneol 15%, α-terpineol 8%, and limonene 1.5% as the main antimicrobial compounds. **Tia et al. (2013)** reported in their study terpinolene 29.21%, α-terpinene 22.55%, 2-γ-carene 8.53% and α-phelandrene 7.61% as the main components of *M. leucadenron* essential oil. **Fall et al. (2017**) identified the 1.8-Cineol, α-Terpineol and β-Citronellol as main components *M. leucadendron* .

The main volatile compounds of the analyzed ABEO based on reduced percentages were valerianol 23.24%, guaiol 16.56%, elemol 9.62%, and γ-eudesmol 7.95% **(Table 3)**.

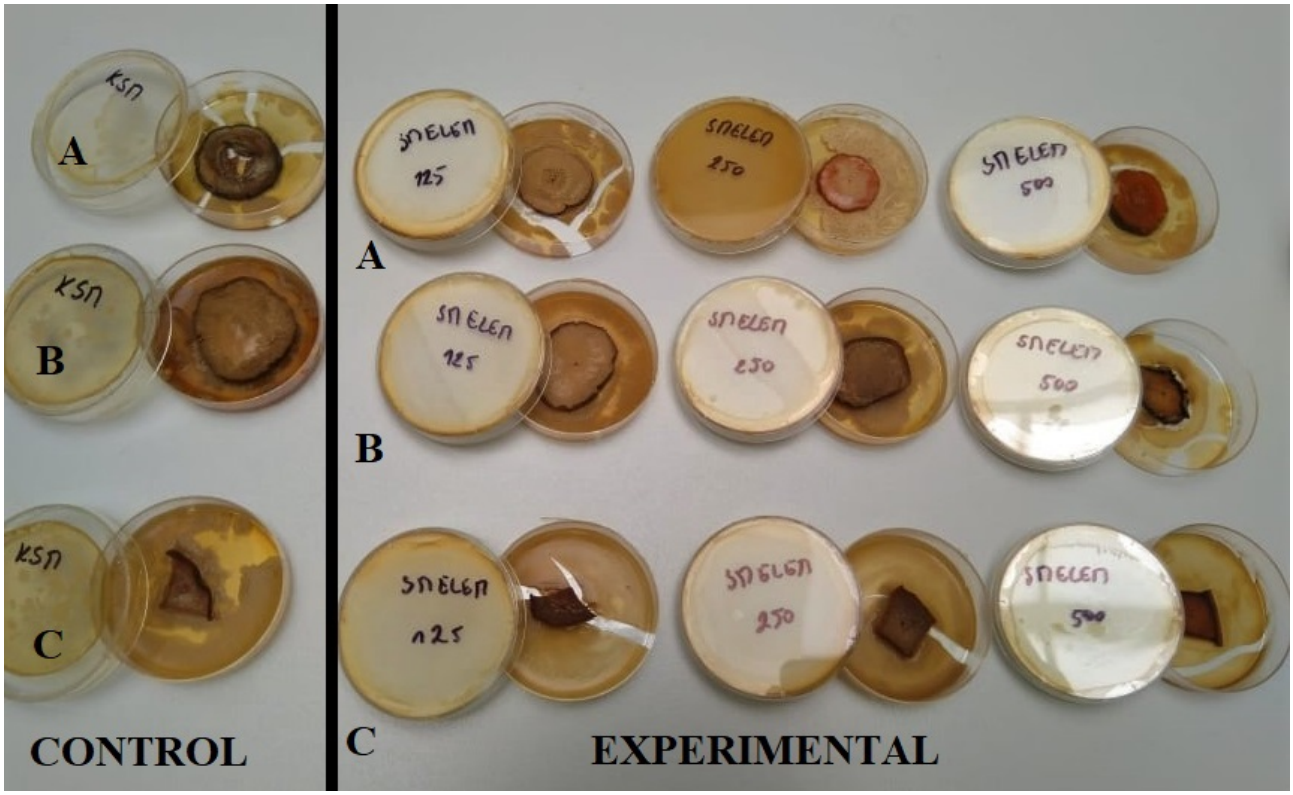

Figure 1 *In situ* antimicrobial analyses of vegetables and fruit with *S. maltophilia* in vapor phase with essential oil *C. luzonicum* (A-carrot, B-potato, C-apple).

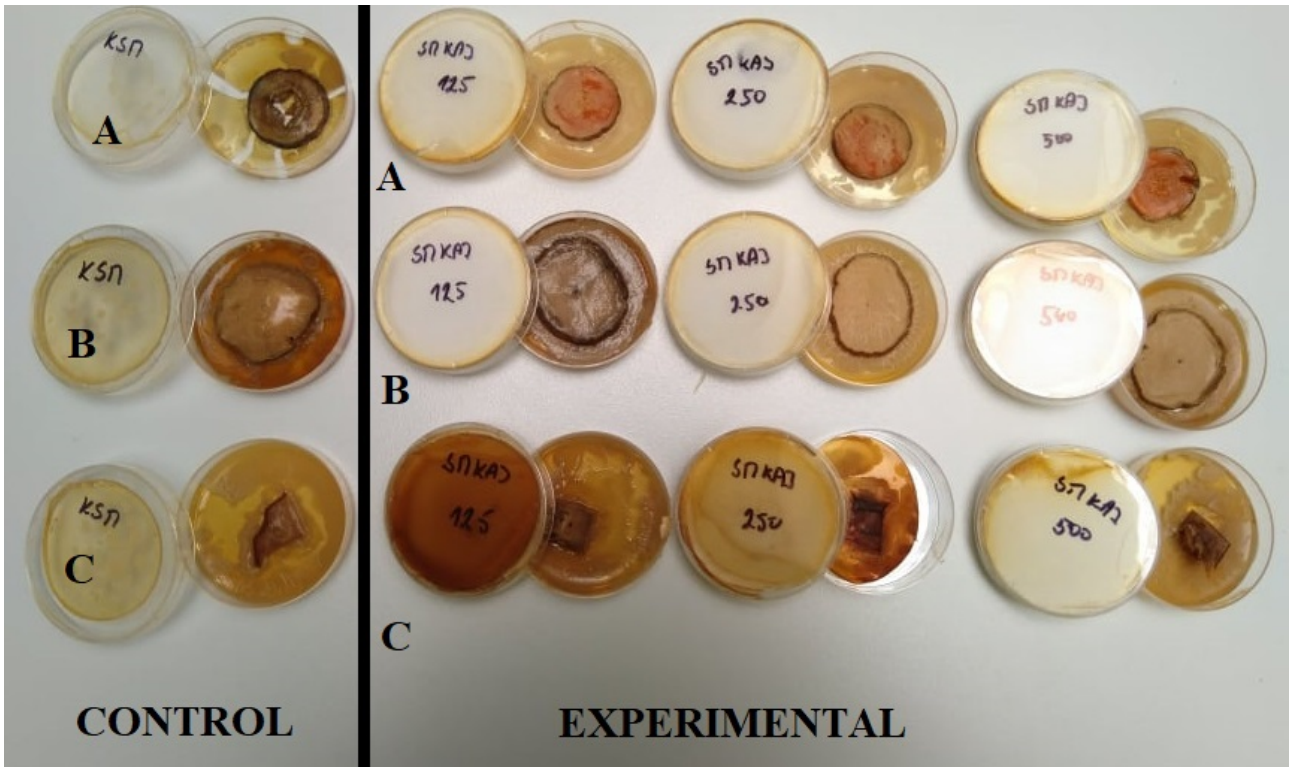

Figure 2 *In situ* antimicrobial analyses of vegetables and fruit with *S. maltophilia* in vapor phase with essential oil *M. leucadendron (*A-carrot, B-potato, C-apple).

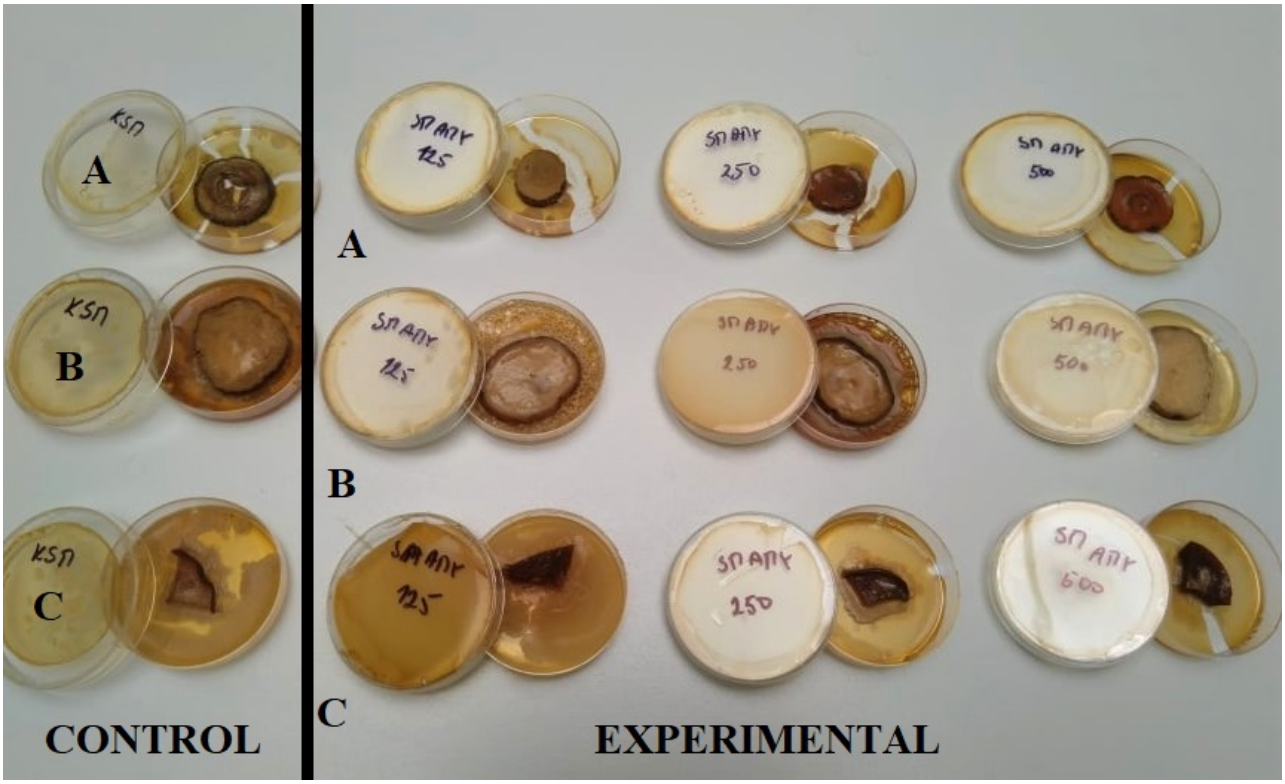

Figure 3 *In situ* antimicrobial analyses of vegetables and fruit with *S. maltophilia* in vapor phase with essential oil *A. balsamifera (*A-carrot, B-potato, C-apple).

Alves et al. (2015) analyzed the chemical composition of amyris essential oil (*Amyris balsamifera* L.) in their study. The main components are eudesmol 23.6%, elemol 14%, and valerianol 12.3%. **Uniyal et al. (2016)** studied the chemical composition of essential oils by gas chromatography and mass spectrometry. Their results show that the main components of amyris oil are β-cadinene 22.66%, (+) - calarene 23.29%, driminol 24%, and linalool oxide 8.48%. **Park and Park (2012)** reported that the major compounds of *A. balsamifera* are β-sesquiphellandrene, elemol, γ-eudesmol and valerianol. **Yun et al. (2012)** they determined chemical compounds essential oil A. balsamifera and the major constituents were elemol, γ-eudesmol and β-sesquiphellandrene.

Antioxidant Activity of essential oils

CLEO essential oil in our study had an antioxidant activity of 33.43%. **Murthy et al. (2016)** determined a DPPH radical inhibition value for the essential oil of 28%. **Lin et al. (2009)** found the antioxidant activity of *C. luzonicum* 11.38%.

MLEO inhibited the DPPH radical at 18.43%. **Pino et al. (2010)** reported that *M. leucadenron* essential oil achieved a free radical inhibition value of 19.9%. **Zhang et al. (2017)** reported antioxidant activity of *M. leucadenron* of 15.7%.

ABEO essential oil achieved an inhibition value of 9.29%. **Nikšić et al. (2018)** studied amyris essential oil in their study and recorded an antioxidant activity of 10.8%. **Dahiya and Manglik (2013)** determined the antioxidant activity of *A. balsamifera* at 19.89%.

The essential oils tested were statistically significantly different (p <0.0001).

Minimum Inhibitory Concentration (MIC)

We determined the MIC 50 and 90 CLEO for *S. maltophilia* to be 6.67 μL.mL^{-1} respectively 6.81 μL.mL^{-1} by using an agar microdilution method. **Nikolic et al. (2016)** focused on the inhibition of clinical isolates of the genus *Candida* by the influence of essential oils and found out that the MIC for *C. luzonicum* oil was 2.5 mg/ml. **Zhang et al. (2017)** reported MIC *C. luzonicum* for *E. coli* 10 μL.mL^{-1} and *P. fluorescens* 12.3 μL.mL^{-1}. **Angelini et al. (2019)** determined MIC of *C. luzonicum* for *A. tubingensis* 12.7 μL.mL^{-1} and *F. oxysporum* 3.17 μL.mL^{-1}.

There was minimum inhibitory concentration 50 and 90 MLEO for *S. maltophilia* 8.25 μL.mL^{-1} and 8.96 μL.mL^{-1}. **Siddique et al. (2020)** reported in his study for *M. leucadendron* MIC values of 4 μL.mL^{-1} for *B. spizizenii*, 8 μL.mL^{-1} *S. aureus* and resistance to *P. aeruginosa* 250 μL.mL^{-1} and *S. enterica* 250 μL.mL^{-1}. **Lieu et al. (2018)** examined the antifungal activity of *M. leucadendron* in food storage and found out MIC of 20 μL.mL^{-1} for *A. niger*. **Pintas and Quave (2019)** focused on the antifungal activity of essential oils against *Malassezia* spp. They determined a MIC of 64 μL.mL^{-1} for *M. leucadendron*. **Bautista-Silva et al. (2020)** found MIC of *M. leucadendra* for *Salmonella thiphymurium* 7.8 μL.mL^{-1} and *Pseudomonas aeruginosa* 31.2 μL.mL^{-1}.

We determined a MIC 50 and 90 of 10.31 μL.mL^{-1} respectively 10.73 μL.mL^{-1} for ABEO. **Xiao et al. (2020)** studied the essential oils and their activity against the stationary phase of *S. aureus*. They determined the MIC for the essential oil of *Amyris balsamifera* 1.5 μL.mL^{-1}. **Santiago et al. (2018)** examined the antibiofilm activity on *Xylella fastidiosa* and found out that the MIC for *Amyris balsamifera* was 125 μL.mL^{-1}.

In Situ Antimicrobial Effect on Vegetables and Fruit

The antimicrobial study of essential oils was determined by an *in situ* method. CLEO inhibited the growth of *S. maltophilia* on the surface of carrots and potatoes at a concentration of 250 mg/ml. Inhibition for apple was recorded at a concentration of 125 mg.mL^{-1} **(Figure 1)**.

MLEO showed inhibition of *S. maltophilia* growth on carrots at a concentration of 500 mg.mL^{-1}, on potato and apple surface at 250 mg.mL^{-1} **(Figure 2)**.

ABEO inhibited bacterial growth at a concentration of 125 mg.mL^{-1} per carrot, 250 mg.mL^{-1} per apple and up to 500 mg.mL^{-1} per potato **(Figure 3)**.

CONCLUSION

The results of our work demonstrated the inhibitory effect of essential oils on *S. maltophila* in a food model. CLEO proved to be the best with the lowest MIC of 6.67 µL.mL^{-1} and antioxidant activity of 33.43% among the tested essential oils. It showed inhibition of *S. maltophilia* growth in the food model at the lowest concentrations among the essential oils.

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THE QUALITATIVE PARAMETERS OF POTATO TUBERS IN DEPENDENCE ON VARIETY AND DURATION OF STORAGE

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ABSTRACT

The qualitative parameters of the potatoes tubers may be significantly changed depending on the variety, group of ripening, way, regime, and duration of storage. So, these researches aimed to define the influence of the variety characteristics and duration storage on qualitative parameters of tuber potatoes. Five potato varieties of two ripening groups were used for testing: medium-early (Satina, Red Lady, Mozart) and medium-ripe (Aroza, Sifra). The chemical properties such as dry matters (DM), starch, sugars (total and reducing), crude protein (CP), ascorbic acid (AA), and nitrates content were determined before and after 2, 4, and 6 months of storage. DM lost during the storage of potato tubers, especially during the period from 4 to 6 months. The group of ripening did not influence this index. Potato tubers of all varieties had high starch content and the duration of storage had a light effect on the level of its losses. In the medium-early group, maximum changes of starch were 1% but in the medium-ripe – 1.9%. A strong effect on the quantities of total sugars (TS) had varietal characteristics of the potato but a group of ripening had light influence. The highest content of sugars was Satin (0.65%) and the smallest Sifra (0.22%) and Mozart (0.23%). After 6 months of storage content of TS depending on the variety increased from 2 till 5 times, while the reducing sugars (RS) increased at least five times and at the end of storage were 0.4% to 0.65%. The amount of AA and its losses during storage depended on the variety. The relative losses during the total storage period were 21.9% in the medium-early group and 28.1% in the medium-ripe. Influence ripening group on level changing AA was not detected. Nitrates' content did not exceed the maximum permissible level (250 mg.kg^{-1}). Their quantities after 6 months were 47 – 61 mg.kg^{-1}.

Keywords: potato; variety; ripening group; storage; qualitative parameters.

INTRODUCTION

The biochemical composition of potato tubers is an indicator of its nutritional value and culinary properties **(Amber, Chegeni and Ferruzzi, 2018)**, which depends on variety, soil, climate, the technology of crop growing, duration and storage conditions **(Nourian, Ramaswamy and Kushalappa, 2003a; Nourian, Ramaswamy and Kushalappa, 2003b)**.

The valuable of potatoes as food is determined by the favorable biochemical composition, which is represented by DM, starch, sugars, CP, and AA. Other components that can affect its nutritional quality are present in smaller quantities in potatoes **(Amber, Chegeni and Ferruzzi, 2018)**.

The content of DM in potatoes can fluctuate within wide enough ranges of 15 – 32% and depends on both varietal characteristics and agro-climatic factors. Their quantities influence the energy value of potatoes and their culinary properties – taste, structure, consistency, and color of pulp after cooking. The quantities of DM decreasing due to physiological processes that take place in the potatoes tubers during the storage **(Zgórska and Grudzińska, 2012; Gunko and Yakovlev, 2016)**.

The basis of DM in potatoes (70 – 80%) presented by starch. Its quantity defines the nutritional value of potato tubers. The amount of starch depends primarily on variety, growing conditions, and has ranged from 9 to 24% by raw weight in the different varieties **(Liu, et al., 2007; Lu, et al., 2011; Ngobese, et al., 2017)**.

Late-ripening potato accumulates more starch substances. The storage process is accompanied by the constant transformation of starch to sugar and vice versa and as a result physiological processes that take place, their quantity was decreasing **(Mareček, et al., 2016)**.

The biological value of potatoes is determined by the content in the tubers of CP. Its biological value is superior compared to the protein of most crops because it contains all the irreplaceable amino acids that are not synthesized in

humans and has an index of valuability is 60 – 92 **(Bártováa, et al., 2009)**.

On average, potatoes contain CP about is 1.9 – 2.2% by raw weight. In the processing of potatoes, protein value is not taken into account in our time, however, in the future, they will take into account the nutritional and physiological value and pass mainly to the processing of potatoes, which are rich in protein. The process of storage is accompanied by quantitative changes in the CP: initially, its amount increases and becomes maxima in March but further storage decreases its content **(Järvan and Edesi, 2009)**.

Freshly harvested potatoes are characterized by low content of sugar: an average about is 0.7% by raw weight or 2.8% by weight of DM. More than half of them are glucose (65%), about 30% – sacharose and only 5% fructose. In the outer and inner layers of tubers the TS content is almost the same, but in the outer layers is presented sacharose and in the central part – monosaccharides. The process of storage escorted constantly transforms starch-to-glucose and vice versa **(Amjad, et al., 2019)**.

As a result, potatoes germination in their tubers may accumulate a large quantity of phosphorus esters of sugar and when there is an intense decomposition of starch to form up to 1% maltose. Increasing the number of sugars (glucose) – is undesirable, as it affects the culinary properties of potatoes, especially the quality of fried products **(Camire, Kubow and Danielle, 2009; Amjad, et al., 2019)**.

Tubers of potatoes contain different vitamins: thiamine, riboflavin, pantothenic acid, pyridoxine, nicotinic acid, carotenoids, and AA but only vitamin C which has content in the ranges from 5 till 40 mg% by raw weight has biological value. Other vitamins presented in quantities very smaller than needed for the health of a person **(Love and Pavek, 2008)**.

The highest content of AA is presented in fresh harvest potato tubers. During storage, their content decreases, especially as a result of germination **(Külen, Stushnoff and Holm, 2013)**.

Potatoes of two groups of ripening – medium-early and medium-ripe were used in the investigations. The choice of potato varieties from these two groups explained by their prevalence in the area, the increase in active temperatures in June, July, and August, low rainfall in the current period and, the need for the stable provision of large cities in the summer and autumn **(De Temmerman, Hacour and Guns, 2002; Hijmans, 2003)**.

Early planting time contributes to large and stable potato harvests in the Forest-Steppe zone of Ukraine **(Voitsehivskyi, et al., 2019)**.

This work aimed to investigate the effect of storage duration, variety characteristics, and ripeness group on changes in the biochemical composition of potato tubers.

Scientific hypothesis

Potatoes are characterized by large quantities of varieties that differ from each other, both in terms of the growing season and biochemical composition. The quality of potatoes tubers changes during storage. The magnitude of these changes depends on the characteristics of soil, climatic conditions, cultivation technology, varietals' characteristics, the activity of physiological processes, conditions, and duration of storage.

MATERIAL AND METHODOLOGY

Material

Materials of the study were potatoes tubers 5 varieties companies HZPC (Netherlands) and Solana (Germany) which apply to two groups of ripeness: medium-early (Satina – control, Red Lady, Mozart) and medium-ripe (Aroza – control, Sifra) **(Figure 1)**. Potatoes were stored in a specialized fridge chamber which equipment by ventilation. Conditions of storage: temperature + 2 to +4 ºC, air humidity 90 – 95%, ventilation – three-volume of air during one day. A sample of 25 kg of potatoes tubers each cultivar was stored in these conditions. From this sample of 1.5 – 2 kg, tubers were taken for the determination of biochemical parameters.

Methodology

Potatoes were grown during 2016 – 2018 years in the conditions of LLC Biotech LTD (Kyiv region, Boryspil district, Horodyshche village), which is located in the Forest-Steppe zone of Ukraine. The total area size of the field for potatoes cultivation is 300 m^2, the accounting area is 240 m^2, the repeatability – 3 times. The potato was cultivated by a common methodology for this crop in the Forest-Steppe zone of Ukraine.

In potato tubers, before storage and after 2, 4, and 6 months were determined DM, starch, TS, RS, CP, AA, and nitrates. The following chemical analyses were performed:

1. Content of DM was performed by the weighted method **(Skaletska, Podpryatov and Zavadska, 2014; DSTU 7804, 2015)**;
2. Content of starch was performed polarimetrically by Ewers **(Savchuk, at al., 2005)**;
3. Content of AA – to restore the Tillmans reagent, by extraction acid solution of sample potato followed by filtration of the resulting substrate titrimetric method according to the state standard of Ukraine 7803 **(DSTU 7803, 2015)**;
4. Content of TS and RS was performed by photocolorimetric method on the photo calorimeter KFK-3-01 according to the state standard of Ukraine ISO 4954 **(DSTU ISO 4954, 2008)**;
5. Content of CP was performed according to the state standard of Ukraine 7169 **(DSTU 7169, 2010)**;
6. The content of nitrates was performed by potentiometry according to the state standard of Ukraine 4948 **(DSTU 4948, 2008)**.

Statistic analysis

All experiments were carried out in triplicate and standard deviations for replication were calculated. The results were statistically analyzed using analysis of variance (ANOVA). Measurements of duplicate samples were expressed as means ± standard deviation. The level of significance associated with the statistical test was 0.05.

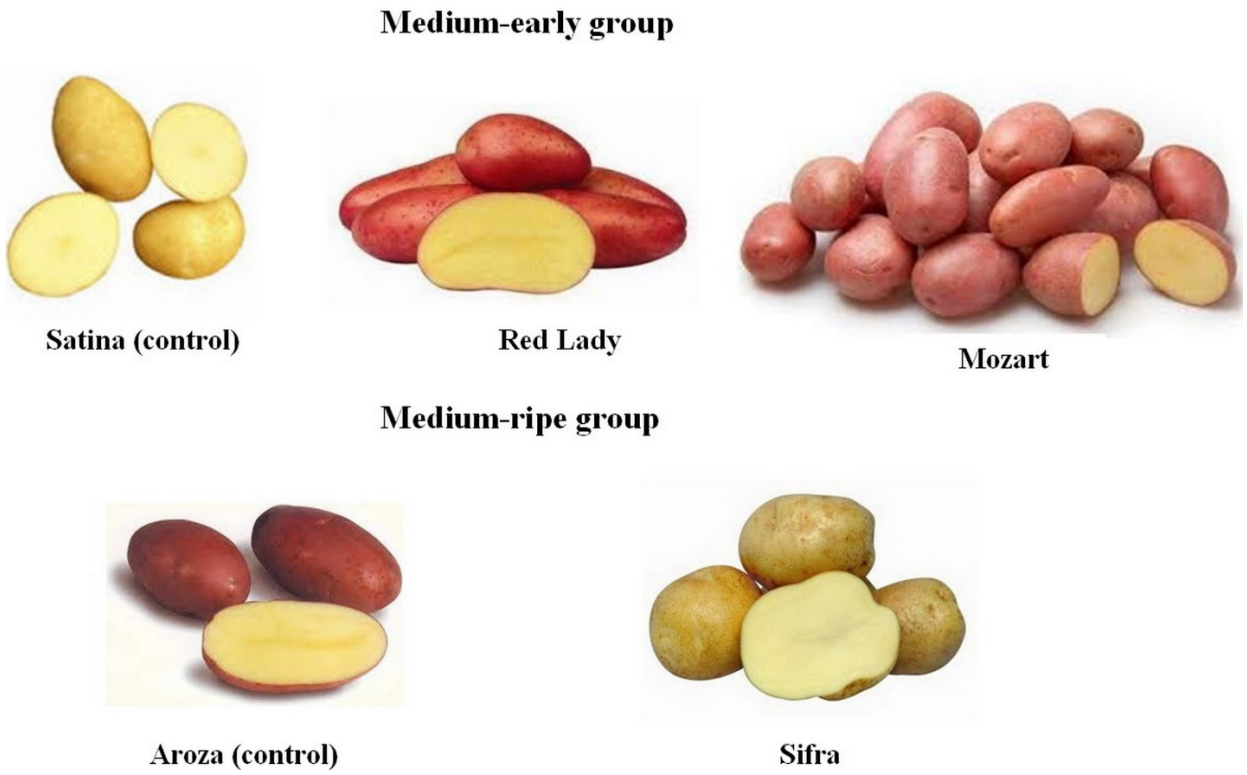

Figure 1 Pictures of potato tubers two groups of ripening.

RESULTS AND DISCUSSION

The biochemical indices of the potato tubers were determined before storage and after 2, 4 and 6 months. The dynamics changes of DM in potato tubers in the experimental varieties were presented in **Table 1**.

According to the research of L. N. Kozlova **(Kozlova, 2005)** on the level of DM accumulation in potato tubers is mainly influenced by the variety of potato (their influence is 46%**)**, the interaction of weather conditions and the place of cultivation – 29% and the combination of these factors – 12%. Other researchers indicated that the most important factors influencing the accumulation amount of dry matter in the potato tubers are varietal characteristics **(Wurr, Beanand Allen, 1978; Mareček, at al., 2015)**, fertilizers and their composition **(Kleinkopf, Westermann and Dwelle, 1981; Pobereźny and Wszelaczyńska, 2011)** and growing zone **(Dinesh, et al., 2005)**.

Our results show that during the storage of tubers potatoes of experimental varieties there was a decrease in the amount of DM, but the intensity of their change was different.

The ripeness group had a significant effect on DM accumulation. Thus, on average in the group of medium-early, at the beginning of storage, the DM content was – 23.5%, and in the group of medium-ripe – 29.3%.

At the end of storage average values of DM depend on the group were 21.1% and 25.9%, respectively. Relative losses in the two groups had averaged values from 9.8 to 11.7% over the whole period of storage. The difference in losses between the individual varieties was more significant, ranging from 2.1% (Mozart**)** to 3.6 % (Aroza).

Particularly intense was the loss of DM in the third storage period from 4 to 6 months, which can be explained by the intensification of physiological processes in potato tubers in the spring. The effect of the ripeness group on the magnitude of the loss was not established. They were affected by the initial amount of DM: than larger quantities were present before storage therefore they were more lost during the storage period.

The basis of the DM in the potato tubers presented starch **(Šimková, et al., 2013; Bhattacharjee, et al., 2014)**. Its content correlates with quantities of DM and the difference between DM and starch is about 5-9 absolute percent.

In our studies, the average content of starch in the potato tubers, depending on the ripeness group, increased from 16.5% in the medium-early to 23.7% in the medium-ripe **(Table 2)**. This indicator was more dependent on a variety.

The obtained results indicate that the index of starch in the tubers varieties which apply to medium-ripe groups sharply differ: from 21.8% (Sifra**)** to 25.5% (Aroza) **(Table 2)**. In the medium-early group, this difference was smaller – from 15.5% (Mozart**)** to 17.7% (Red Lady).

Storage for 6 months did not cause significant changes in the starch of the tubers. Thus, during three years of research an average of the relative losses were: in the medium-early group – 1% but in the group medium-ripe – 1.9%. The high starch content after 6 months of storage had the varieties of Aroza (23.3%) and Sifra (20.1%), which makes it possible to recommend these varieties for processing with obtaining starch and alcohol.

The content of CP in potato tubers was from 1.9% to 2.2% by weight and more than half of its total content was protein **(Vlasyuk, Vlasenko and Mitsko, 1979)**. The amount of protein that accumulation in potato tubers mainly depends on agro-ecological conditions of cultivation **(Bártová, et al., 2009)**, fertilizers, and their composition **(Ahmed, et al., 2009; Petropoulos, et al., 2020)**.

Table 1 Dynamics of DM in potato tubers, %.

Variety	Before storage	Duration of storage, days		
		60	120	180
		medium-early		
Satina – control	23.1 ±0.11*	22.6 ±0.32*	21.9 ±0.47*	20.8 ±0,34*
Red Lady	25.1 ±0.05*	24.3 ±0.31*	23.6 ±0.35*	22.4 ±0,28*
Mozart	22.2 ±0.31*	21.7 ±0.26*	21 ±0.45*	20.1 ±0,33*
		medium-ripe		
Aroza – control	31.1 ±0.22*	30.2 ±0.21*	29 ±0.22*	27.5 ±0,29*
Sifra	27.4 ±0.23*	26.6 ±0.28*	25.5 ±0.33*	24.2 ±0,31*

Note: * denotes statistically significant difference at $p < 0.05$ level.

Table 2 Dynamics of starch substances in potato tubers, %.

Variety	Before storage	Duration of storage, days		
		60	120	180
		medium-early		
Satina – control	16.3 ±0.26*	16.2 ±0.22*	15.9 ±0.34*	15.4 ±0.48*
Red Lady	17.7 ±0.26*	17.5 ±0.26*	17.0 ±0.26*	16.3 ±0.34*
Mozart	15.5 ±0.2*	15.4 ±0.27*	15.2 ±0.43*	14.8 ±0.38*
		medium-ripe		
Aroza – control	25.5 ±0.26*	25.1 ±0.33*	24.4 ±0.34*	23.3 ±0.22*
Sifra	21.8 ±0.34*	21.6 ±0.23*	21.0 ±0.3*	20.1 ±0.45*

Note: * denotes statistically significant difference at $p < 0.05$ level.

Table 3 Dynamics of CP in potato tubers, %.

Variety	Before storage	Duration of storage, days		
		60	120	180
		medium-early		
Satina – control	1.8 ±0.06*	1.9 ±0.15*	1.9 ±0.2*	2.0 ±0.18*
Red Lady	1.5 ±0.13*	1.5 ±0.11*	1.7 ±0.13*	1.8 ±0.2*
Mozart	1.7 ±0.06*	1.9 ±0.1*	2.0 ±0.13*	2.1 ±0.15*
		medium-ripe		
Aroza – control	2.0 ±0.07*	2.0 ±0.16*	2.1 ±0.11*	2.2 ±0.14*
Sifra	1.6 ±0.13*	1.6 ±0.06*	1.7 ±0.14*	1.9 ±0.11*

Note: * denotes statistically significant difference at $p < 0.05$ level.

Analysis of the content of CP indicated the low difference between these indexes between two groups of maturity **(Table 3)**.

An average during three years of research, its quantity was in the group of medium-early – 1.7% and in the group of medium-ripe – 1.8%. The highest content of CP was observed in the tubers of the 2016 harvest, which confirms its dependence on from conditions of the growing season. The obtained results are confirmed by conclusions of other researchers **(Eliseeva, 1996)**, that indicate the influence of rainfall, temperature conditions during vegetation on the accumulation of CP.

In the process of storage of tubers potato of experimental varieties, the content of CP increased and especially intensively in the period from 4 to 6 months **(Table 3)**. This result is in agreement with the results of the researches obtained by S.F. Polishchuk, which indicates that the maximum content of CP in tubers accumulates in March **(Polishchuk, 1986)**.

The sugar content of the potatoes can vary greatly depending on the variety, the technology of cultivation (fertilizers), the state of the tubers and the storage conditions **(Dogras, Siomos and Psomakelis, 1991; Ohara-Takada, et al., 2005; Galdón, et al., 2010; Muttucumaru, et al., 2013; Mareček, et al., 2013)**. Young tubers of potatoes have more sugar than ripening tubers. Tubers that have higher sucrose content than monosaccharides and have better keeping capacity **(Sowokinos, 1978; Richardson, Davies and Ross, 1990)**.

The highest importance on the ability to accumulate monosaccharides as a result of starch hydrolysis at low storage temperatures in the tubers has varietal features. Thus, the researchers recorded an increase in the sugar content in the potato tubers of the Yantarniy variety from 0.55% to 3.48% during storage at low temperatures, which made them unsuitable for industrial processing **(Gusev and Metlitsky, 1982)**.

In our investigations, the TS content an average during three years of research was 0.43% in the medium-early group and 0.4% in the medium-ripe group **(Table 4)**.

Thus, on average, during the three years of investigation, the content of TS did not differ significantly between the ripeness groups but had differences between the varieties. The highest content of sugars was contained in the potatoes of the Satina variety (0.65%) and the lowest in the Sifra (0.22%) and Mozart (0.23%) varieties. The content of RS in potatoes ranged from 0.07% to 0.28%, which makes them suitable for industrial processing **(Table 5)**.

The storage process was accompanied by an increase in the content of both total and reducing sugars.

Table 4 TS dynamics in the potato tubers, %.

Variety	Before storage	Duration of storage, days		
		60	120	180
		medium-early		
Satina – control	0.65 ±0.2*	0.97 ±0.12*	1.16 ±0.04*	1.37 ±0.07*
±Red Lady	0.41 ±0.21*	0.73 ±0.14*	0.85 ±0.05*	1.17 ±0.05*
Mozart	0.23 ±0.3*	0.55 ±0.26*	0.78 ±0.04*	1.12 ±0.03*
		medium-ripe		
Aroza – control	0.58 ±0.23*	0.94 ±0.2*	0.96 ±0.03*	1.31 ±0.06*
Sifra	0.22 ±0.09*	0.51 ±0.02*	0.78 ±0.05*	0.92 ±0.03*

Note: * denotes statistically significant difference at $p < 0.05$ level.

Table 5 Dynamics of RS in potato tubers, %.

Variety	Before storage	Duration of storage, days		
		60	120	180
		medium-early		
Satina – control	0.28 ±0.08*	0.43 ±0.04*	0.51 ±0.02*	0.65 ±0.06*
Red Lady	0.18 ±0.02*	0.3 ±0.02*	0.37 ±0.04*	0.52 ±0.04*
Mozart	0.09 ±0.01*	0.21 ±0.03*	0.35 ±0.03*	0.47 ±0.04*
		medium-ripe		
Aroza – control	0.26 ±0.01*	0.39 ±0.04*	0.44 ±0.07*	0.58 ±0.04*
Sifra	0.07 ±0.01*	0.22 ±0.02*	0.33 ±0.04*	0.4 ±0.02*

Note: * denotes statistically significant difference at $p < 0.05$ level.

Table 6 Dynamics of AA in potato tubers, %.

Variety	Before storage	Duration of storage, days		
		60	120	180
		medium-early		
Satina – control	8.7 ±0.13*	8.2 ±0.33*	7.5 ±0.2*	6.7 ±0.14*
Red Lady	9.1 ±0.33*	8.7 ±0.28*	8.4 ±0.23*	7.5 ±0.17*
Mozart	11.4 ±0.14*	11.0 ±0.29*	10.3 ±0.21*	8.5 ±0.12*
		medium-ripe		
Aroza – control	13.4 ±0.26*	12.5 ±0.21*	11.7 ±0.23*	9.3 ±0.1*
Sifra	9.8 ±0.23*	9.5 ±0.27*	9.1 ±0.27*	7.3 ±0.08*

Note: * denotes statistically significant difference at $p < 0.05$ level.

Table 7 Dynamics of nitrates in potato tubers, $mg.kg^{-1}$.

Variety	Before storage	Duration of storage, days		
		60	120	180
		medium-early		
Satina – control	74 ±2.32*	68 ±4.17*	63 ±2.67*	56 ±2.35*
Red Lady	85 ±2.66*	76 ±3.35*	72 ±3.12*	61 ±2.15*
Mozart	63 ±2.13*	57 ±2.67*	51 ±4.63*	47 ±2.41*
		medium-ripe		
Aroza – control	71 ±2.44*	64 ±2.56*	57 ±3.35*	49 ±2.83*
Sifra	80 ±4.31*	73 ±3.31*	65 ±3.34*	52 ±2.67*

Note: * denotes statistically significant difference at $p < 0.05$ level.

During the whole storage period, the TS content increased depends on variety from 2 to 5 times but RS increased at least five times and at the end of storage (after 6 months**)** we obtain from 0.4% to 0.65%.

That is, most varieties were characterized by an excess of optimum content of sugar 0.2 – 0.4%, which makes them unsuitable for the production of fried potatoes. However, some researchers indicate **(Metlitsky, Gusev and Tektonidi, 1972; Marquez and Añon, 1986)** that a significant deterioration in the color of potato products appears only with the content of RS is 1% and more. Some researchers **(Roe, Faulks and Belsten, 1990)** indicated the effect of fertilizers, especially nitrogen, on the darkening of potatoes during frying.

The amount of vitamin C that accumulates in potato tubers depends on many factors, the main of which are varietal characteristics, fertilizers and their doses, farming systems, zones of cultivation, soil and climatic conditions **(Hamouz, et al., 2009; Macák, Žák and Polláková, 2012)**.

Before storage, the mean content of AA in potato tubers was 9.7% in the medium-early group and 11.6% in the medium-ripe group **(Table 6)**.

The process of potatoes storage was accompanied by a decrease in the amount of AA, which negatively affected the biological value. Its relative losses during the storage

period an average set of 21.9% in the medium-early group and 28.1% in the medium-ripe group, respectively.
The lost of AA depends mainly on varietal characteristics. According to our research during the whole storage period, the greatest losses had potatoes of Aroza variety (30.6%) and the lowest – Red Lady (17.6%).

The content of nitrates in the product determines the level of its safety and is regulated according to sanitary standards. The high content of nitrates presented in the skin of the tuber and the areas of the eyes **(Ilchuk, 1993)**. As a result preparation of French fries and dried potatoes, the content of nitrates reduced by 70 – 80 % but chips by 72 – 76% **(Zabara, 2000)**. The quantity of nitrates in the potato tubers depends on different factors, the main of which are fertilizers and their doses, farming systems, and type of soil **(Yli-Halla, Viikari and Palonen, 1987; Ahmed, et al., 2009)**.

In our experiments, the nitrate content before storage was lower than allowed according to the maximum permissible level and ranged from 63 to 85 $mg.kg^{-1}$ but at the end of storage were 47 – 61 $mg.kg^{-1}$ **(Table 7)**. That is, the relative losses of nitrates during the whole storage period an average were from 25 to 28 %.

CONCLUSION

The results of the effect of storage duration, variety characteristics, and ripeness group on changes in qualitative parameters of potato tubers were established.
The potato tubers of the experimental varieties accumulated different amounts of DM. Their quantity depended on the length of the growing season, that is, the medium-early ones accumulated less (23.5%) and the medium-ripe ones more (29.3%). The process of storage of tubers was accompanied by the loss of DM, especially in the period from 4 to 6 months that explained by the intensification of physiological processes in potatoes in the spring. The effect of the ripeness group on the magnitude of the DM loss was not established.

All potato varieties had a high content of starch. Storage and group of ripening did not significantly affect the level of its losses (medium-early – 1%; medium-ripe – 1.9%). The highest starch content after 6 months of storage characterized two varieties of Aroza (23.3%) and Sifra (20.1%).

The amount of CP in the potato tubers for three years depended on the conditions of the growing season. This indicator increased during storage. Its maximum value we obtain after 6 months and the magnitude of growth over an average of three years in both maturity groups was 0.25 – 0.26%.

The amount of TS depended on the varietal characteristics of the potato: the highest content was characterized by the variety Satin (0.65%) and the lowest – Sifra (0.22%) and Mozart (0.23%). During the whole storage period, the TS content increased depending on the variety from 2 to 5 times, but RS was by 5 times and more.

The amount of AA and its loss during storage depended on the variety. During the storage period, relative losses on average were 21.9% in the medium-early group and 28.1% in the medium-ripe group. The largest losses were potatoes of Aroza (30.6%) and the smallest – Red Lady (17.6%).

Potato tubers had nitrate content less than allowed by the maximum permissible level and during storage, their relative losses were 25 to 28%.

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MONITORING OF MICROSCOPIC FUNGI COMMUNITY IN SELECTED BEE PRODUCTS

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ABSTRACT

Honey is a remarkably complex food with a valued place in the human diet. An important indicator of its quality is the presence of microorganisms. This study aimed to monitor the mycological quality of 27 samples of Slovak kinds of honey and honey products with the addition of differently processed blueberries, cranberries, and red currants. Yeast and filamentous microscopic fungi were monitored using the plate dilution method. A total of 21 samples (78%) were positive for the presence of yeasts and 14 samples (52%) were positive for the filamentous microscopic fungi occurrence. In 6 samples (22%) no presence of microscopic fungi was found at all. The highest number of yeasts (3.07 log CFU.g^{-1}) was recorded in one flower honey sample and in other samples, yeast counts did not exceed 3 log CFU.g^{-1}. The highest numbers of filamentous micromycetes (2.39 and 2.44 log CFU.g^{-1}) were recorded in 2 honeydew honey samples. Overall, the following genera have been identified: *Alternaria*, *Arthrinium*, *Aspergillus* (including previously named as *Eurotium*), *Aureobasidium*, *Cladosporium*, *Mucor*, *Penicillium,* and *Stemphilium*. *Penicillium* spp. were recorded with the highest isolation frequency (41%). *Aspergillus* species were isolated from 19% of honey samples. In the honey with fruit addition, the yeasts in a range of 1.00 – 3.09 log CFU.g^{-1} and the filamentous microscopic fungi in a range of 1.00 – 1.39 log CFU.g^{-1} were found. The study showed that cranberries were the most appropriate addition from a mycological point of view. Dried and lyophilized forms of tested fruits were the most suitable. Except for honey with frozen currants and honey with fresh cranberries, all final products had a water activity below 0.610 and appeared to be stable.

Keywords: blueberry; cranberry; honey; micromycetes; red currant.

INTRODUCTION

Honey is a remarkably complex food that, contains more than 200 biologically active compounds. The main components of honey are carbohydrates and water. Some minor components present in honey are minerals, phenolic acids and flavonoids, ascorbic acid, proteins, certain enzymes, amino acids, organic acids, α-tocopherol, carotenoids, and Maillard reaction products **(Korošec et al., 2016)**.

Honey is characterized by medicinal effects and antimicrobial properties and is used in various cuisines. The application potential in bakery, confectionery, snack foods, fruit, and vegetable products and beverages is ever-increasing **(Aparna et Rajalakshmi, 1999)**. Nowadays there is an ever-increasing demand for consumer-based diversified honey products, which lead to value addition to the raw material. The honey-based spreads increase the demand for honey and provide superior products to the customers in terms of nutrition and taste **(Umesh Hebbar, Rastogi and Subramanian, 2008)**. Honey can be enriched with various additives such as nuts, well-dried herbs, spices, or fruits **(Dadant, 2019)**. According to **Krell (1996)** for the production of honey with added fruit, it is best to use soft, sun-dried fruits with the lowest possible water content. Fresh fruit can become contaminated and mildewed **(Dadant, 2019)**.

As a therapeutic product, honey has been used since ancient times. Its medical use represents support for the treatment of cold and flu, insomnia, liver and gallbladder diseases, or heartburn. Because honey contains digestive enzymes, it helps reduce excess fat in the human body **(Chaven, 2014)**. Its properties are chemically evidenced by its composition. Among features that make this product effective against microorganisms, we can quote high osmotic pressure by low water activity (average 17.2%); low pH because of the presence of organic acids (average 3.9); the presence of hydrogen peroxide generated by the action of enzyme glucose oxidase; low protein content; low

redox potential due to the presence of reducing sugars; and chemical agents present as lysozyme, phenolic acids, pinocembrin, terpenes, benzyl alcohol, and volatile substances **(Rao et al., 2016; Snowdon and Cliver, 1996)**. Despite all the honey microorganism barriers, some species of microorganisms can survive and may cause damage to honeybees or consumers **(Silva et al., 2017)**. Primary sources of microbial contamination are likely to include pollen, the digestive tracts of honey bees, the environment inside the beehive, dust, air, flowers, nectar, sources that are very difficult to control. Secondary (after-harvest) sources of contamination include air, food handlers, cross-contamination, equipment,and buildings, and these can be controlled by good manufacturing practices **(Snowdon and Cliver, 1996; Olaitan, Adeleke and Ola, 2007)**. Microbes of concern in post-harvest handling are those that are commonly found in honey (i.e., yeasts and spore-forming bacteria), those that indicate the sanitary or commercial quality of honey (i.e., coliforms and yeasts), and those that under certain conditions could cause human or honeybee illness **(Snowdon and Cliver, 1996)**.

Fungi can colonize most of the substrata on Earth. Honey is a reservoir of xerotolerant and xerophilic fungi and should be an ideal substratum for their development. Flowers and aphids could play an important role as a source of such microorganisms. However, little information has been gathered about these fungi and their relationships with honey and honey products. Honey has been studied little in terms of its fungal diversity **(Rodríguez-Andrade et al., 2019)**. Filamentous fungi and yeasts can maintain their vegetative form **(Snowdon and Cliver, 1996)**. They can remain latently in this product waiting for the moment in which the environment is suitable for their development **(Silva et al., 2017)**. They may participate in the fermentation and decay of honey. Yeast activity can cause foaming of the honey surface, smell, and taste of the fermented product **(Přídal, 2005)**. **Soni et al. (2016)** state, that the detection of yeast counts in honey is a complementary factor in determining the quality of honey. Growth of some filamentous fungi could be followed by the production of mycotoxins, which are secondary metabolites of filamentous fungi and toxic to humans and animals even in small concentrations **(Pitt, 2000)**. Among mycotoxin producers, we should highlight *Aspergillus* spp. and *Penicillium* spp. because they are the most commonly found in honey **(Foley et al., 2014; Sinacori et al., 2014)**. They are also associated with diseases in honeybees **(Silva et al., 2017)**.

It is important to know the diversity of contaminating microorganisms in honey, especially due to disseminate pathogenic microorganisms in international traded marketing **(Silva et al., 2017)**. In general, honey should be considered as a "living food" and, consequently, its "normal" mycobiota merits more extensive study **(Rodríguez-Andrade et al., 2019)**.

The aim of the present study was therefore to monitor the mycological quality of Slovak honey and selected honey products with added fruit. The fruit affects the basic Physico-chemical properties of the honey, and it may impact their stability and the development of micromycetes, so we aimed to estimate the safety of these products in terms of the yeast and filamentous microscopic fungi occurrence.

Scientific hypothesis

Honey contains various microorganisms, including microscopic fungi, which may, under certain circumstances, affect its quality and safety.

MATERIAL AND METHODOLOGY

Analyses in the present study were divided into two parts. The first were analyses of honey samples recovered from the Slovak production and the second were analyses of honey with the addition of various fruits. All analyses were focused on the detection of microscopic fungi in the monitored commodities, namely yeasts and filamentous microscopic fungi.

Honey

A total of 27 different honey samples were mycologically monitored (20 samples from the year 2018 and 7 samples from the year 2019). A list of honey samples with their characteristics is given in **Table 1**. All samples were obtained directly from beekeepers.

Each honey sample in the present study was tested for the occurrence of yeasts and filamentous microscopic fungi using a plate dilution method and appropriate culture media.

Honey with the addition of fruit

In the second part of the study, were (under laboratory conditions) prepared samples of honey with the addition of various fruits in various forms and concentrations. The fruits used in this study were blueberries (frozen, dried, and lyophilized), cranberries (fresh, dried, and lyophilized), and red currants (frozen, dried, and lyophilized). **Table 2** provides a more detailed description of the raw materials used to prepare the products. Weighed fruits were mixed with rapeseed pasted honey, particularly in the concentrations shown in **Table 3**, and have been thoroughly homogenized. Concentrations were designed according to the expected water content of the used fruit. With decreasing water concentration in fruits, the content of their addition was increased. The products were stored in glass containers in a cool and dark place. Mycological analyses were performed both on individual raw materials and final products, all variants in 3 repetitions.

Mycological analyses

Mycological analyses of honey samples, fruit samples, and honey samples with additives were aimed at determining the total counts of yeasts and filamentous microscopic fungi. A plate dilution method was used for the above analyses. The procedures were followed according to Slovak technical standards - STN ISO 21527-1 (2010) and STN ISO 21527-2 (2010). Two nutrient media were used according to the water activity of the particular sample analyzed. For samples with water activity greater than 0.95, DRBC nutrient medium (Dichloran Rose Bengal Chloramphenicol Agar; Himedia, M1881) was used.

Table 1 Slovak origin honey samples monitored for the microscopic fungi occurrence.

Sample number	Botanical origin	Geographical origin	Year of production
1	blossom (sunflower)	Dunajská Lužná (Senec)	2018
2	blossom (acacia)	Nitra (Nitra)	2018
3	blossom (linden)	Nitra (Nitra)	2018
4	blossom	Nitra (Nitra)	2018
5	blossom (sunflower)	Nitra (Nitra)	2018
6	honeydew	Nitra (Nitra)	2018
7	blossom (buckwheat)	Rišňovce (Nitra)	2018
8	buckwheat	Dežerice (Bánovce nad Bebravou)	2018
9	blossom (acacia)	Dežerice (Bánovce nad Bebravou)	2018
10	blossom	Malé Kršteňany (Partizánske)	2018
11	blossom	Babín (Námestovo)	2018
12	blossom	Horná Orava (Námestovo)	2018
13	blossom	Dolný Pajer (Žarnovica)	2018
14	blossom	Smrečany (Liptovský Mikuláš)	2018
15	blossom	Smrečany (Liptovský Mikuláš)	2018
16	blossom (spring)	Nová Ľubovňa (Stará Ľubovňa)	2018
17	honeydew	Nová Ľubovňa (Stará Ľubovňa)	2018
18	blossom	Orlov (Stará Ľubovňa)	2018
19	blossom	Orlov (Stará Ľubovňa)	2018
20	honeydew	Orlov (Stará Ľubovňa)	2018
21	blossom (linden)	Nitra (Nitra)	2019
22	honeydew	Nitra (Nitra)	2019
23	honeydew	Nitra (Nitra)	2019
24	honeydew	Dolný Pajer (Žarnovica)	2019
25	honeydew	Dolný Pajer (Žarnovica)	2019
26	honeydew	Žarnovica (Žarnovica)	2019
27	honeydew	Žarnovica (Žarnovica)	2019

Table 2 Characteristics of raw materials used for the production of honey with added fruit.

Sample number	Raw materials	Country of origin	Year of production /min. durability*	Comments
1	honey	Slovakia (Malé Kršteňany)	2017	rapeseed, pasted, without heating, purchased from a beekeeper
2	blueberries – frozen	Slovakia (High Tatras)	2018	obtained by direct harvesting
3	blueberries – dried	unknown	2018	bought in the shopping centrum in Nitra - for the weight
4	blueberries – lyophilized	Chile	June 2020*	ecological agriculture
5	cranberries – fresh	USA	2019*	1st class product
6	cranberries – dried	USA	2019*	1st class product, purchased in an organic shop
7	cranberries – lyophilized	Germany	2020*	whole lyophilized fruits purchased through the e-shop
8	red currants – frozen	Slovakia (Smrečany)	2018	obtained by direct harvesting
9	red currants – dried	Poland	2019*	whole fruits
10	red currants – lyophilized	Germany	2017	whole fruits

Table 3 Concentrations of individual fruits used for the preparation of honey with additives.

Product number	Fruit		Honey quantity [g]	Fruit concentration [%]
	Quantity [g]	Type		
11	2	blueberries – frozen	98	**2**
12	10	blueberries – dried	90	**10**
13	12	blueberries – lyophilized	88	**12**
14	4	cranberries – fresh	96	4
15	10	cranberries – dried	90	10
16	6	cranberries – lyophilized	94	6
17	6	red currants – frozen	94	**6**
18	10	red currants – dried	90	**10**
19	12	red currants – lyophilized	88	**12**

In the case of samples with a water activity less than or equal to 0.95, the DG18 medium (Dichloran 18% Glycerol Agar; Himedia, M1129) was used. Cultivation was performed under aerobic conditions at 25 °C for 5 – 7 days. Grown colonies were converted to colonies forming unit (CFU), using the formula (1)

$$N = \Sigma C/[(1 \times n1) + (0.1 \times n2)]d \quad (1)$$

where N = number of colonies per milliliter or gram, ΣC = sum of all colonies on all plates counted, n1 = number of plates in lower dilution counted, n2 = number of plates in next highest dilution counted, d = dilution from which the first counts were obtained.

Filamentous microscopic fungi were subsequently identified into the genera. The identification was based on the observation of macro- and micromorphological features, using respected mycological keys **(Samson et al., 2002; Pitt and Hocking, 2009; De Hoog, Guarro, and Gene, 2000)**.

Water activity (a_w) determination

The reference device, LabMaster-aw from Novasina, was used to determine the water activity of the raw materials and honey products. The measurements were performed at a constant temperature of 25 °C, in 2 replicates and the average values were taken into account.

Statistical analysis

Counts of microorganisms were reported as mean values calculated from the results of 3 replicates of mycological analyses of each sample. These were then converted to decimal logarithms, referred to as log CFU.g^{-1}.

The obtained mycological results were evaluated and expressed in isolation frequency (Fr), which is defined as the percentage of samples within which the species or genus occurred at least once. These values were calculated according to **González et al. (1996)** using the formula (2):

$$Fr\ (\%) = (ns / N) \times 100 \quad (2)$$

where ns = number of samples within a genus; N = total number of samples.

RESULTS AND DISCUSSION

Mycological analyses of honey samples

In the first part of the study, we analyzed 27 honey samples of Slovak origin. Of these, a total of 21 samples (78%) were positive for the presence of yeasts and 14 samples (52%) were positive for the filamentous microscopic fungi occurrence. In 6 samples (22%) no presence of microscopic fungi was found at all. The counts of detected micromycetes are shown in **Figure 1**. The highest number of yeasts (3.07 log CFU.g^{-1}) was recorded in flower honey sample no. 19. According to **Snowdon and Cliver (1996)**, most samples of honey contain detectable levels of yeasts. Although yeast counts in many honey samples are below 100 CFU.g^{-1}, yeasts can grow in honey to very high numbers. **Bogdanov (2011)** states that water content is an important factor for yeast development in the substrate. Honey generally contains osmophilic (sugar–tolerant) yeasts in greater or lesser amounts. If the moisture content is high enough and storage temperature is favorable, they can ferment substrate **(Subramanian, Umesh Hebbar and Rastogi, 2007)**. Fermentation is an irreversible phenomenon that can run in honey mainly during storage, causing significant economic losses. In such a case, honey presents a characteristic odor, increasing acid flavor and gas bubbles **(Perez-Perez, Rodriguez-Malaver and Vit, 2007)**. **Bogdanov (2011)** expresses the relationship between fermentation risk and water content as follows. If the water content is 17% or less - honey is safe regardless of the number of yeasts. If the water content is 17.1 – 18% - honey is safe for yeasts up to 1000 CFU.g^{-1} (3 log CFU.g^{-1}). If the water content is 18.1 – 19% - honey is safe for yeasts up to 10 CFU.g^{-1} (1 log CFU.g^{-1}). If the water content is 19.1 – 20% - honey is safe for yeasts up to 1 CFU.g^{-1} (<1 log CFU.g^{-1}). And finally, if the water content is over 20% - it is a constant risk of fermentation in honey. Similarly, **Subramanian, Umesh Hebbar and Rastogi (2007)** mention, that raw honey sample containing more than 20% moisture readily undergoes fermentation irrespective of the initial yeast count and reduction of moisture content below 17% is considered to be a safe level for retarding yeast activity. The water content in honey is one of the most important criteria of honey quality. The lower the water content, the more viscous, thick and unchanged honey is since such conditions in honey are unsuitable for the growth of osmophilic yeasts and thus prevent fermentation of honey **(Korošec et al., 2016; Čermáková, Chlebo and Husáriková, 2017)**. A previous study **(Kňazovická et al., 2020)** on the physico-chemical parameters of honey from 2018 (our first 20 samples) concluded that the water content of the honey did not exceed 20% and its average value was 17.3%.

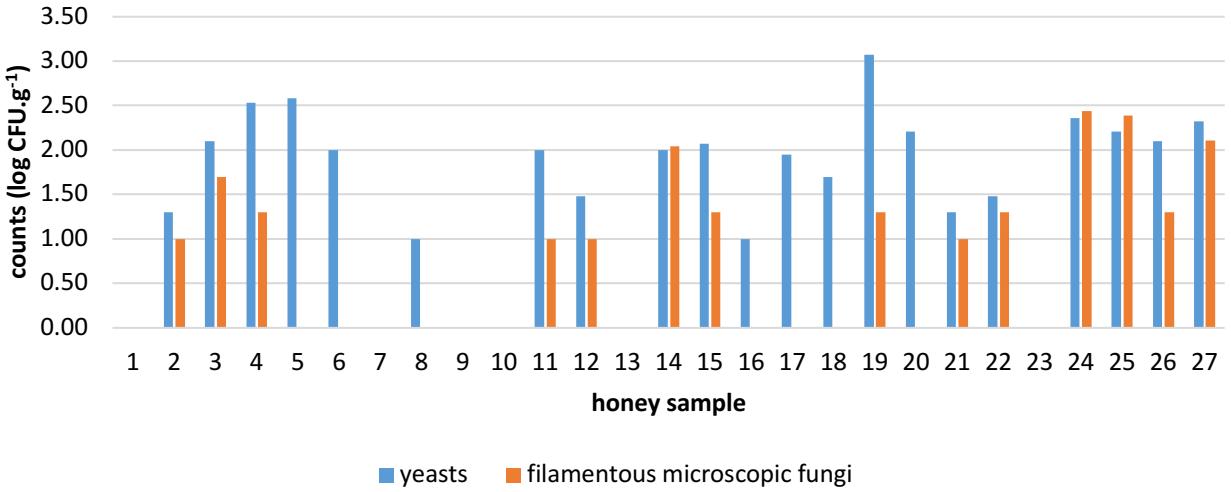

Figure 1 Counts of detected microorganisms in honey samples of Slovak origin.

From this perspective, except for the flower honey sample no. 19, we can consider our honey as safe.

In this study, we did not perform yeast genera identification, but the most common yeasts isolated from honey include for example *Schizosaccharomyces*, *Saccharomyces,* and *Zygosaccharomyces* **(Silva et al., 2017; Tančinová et al., 2017)**. Among the isolated Portuguese honey samples, *Rhodotorula mucilaginosa*, *Candida magnoliae,* and *Zygosaccharomyces mellis* were the predominant species. *Candida* spp. represented more than 45% of the honey isolates **(Carvalho et al., 2010)**. Authors **Sinacori et al. (2014)** gave information about the isolation of *Debaryomyces hansenii*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces mellis*, *Aureobasidium pullulans,* and *Cryptococcus uzbekistanensis* species from honey. Among them, *Cryptococcus* species were associated with human pathogenicity. *Cryptococcus neoformans* is characterized as opportunistic human pathogen, able to infect the central nervous system **(Ashbee and Bignell, 2010)**. **Čadež et al., (2014)** isolated from bee bread and honey in Hungary five yeast strains representing a hitherto undescribed yeast species. The name *Zygosaccharomyces favi* sp. nov. is proposed for this new yeast species, which based on phenotype can be distinguished from related *Zygosaccharomyces* species by its obligate osmophilic nature.

In terms of filamentous micromycetes, the highest numbers we recorded in honeydew honey samples no. 24 and 25 (2.44 a 2.39 log CFU.g^{-1}). Honeydew kinds of honey are generally reported to contain fungal hyphae and algae when microscopically examined because bees collect honeydew, which is the product of aphids inhabiting green parts of plants. Bees along with honeydew collect additional structures, such as hyphae or spores of fungi, plant pathogens, and algae **(Escuredo, Fernández-González and Seijo, 2012)**. However, we have found that honeydew honey does not necessarily show higher numbers of viable filamentous fungi, because in 4 of the 9 honeydew honey samples we did not detect any occurrence of filamentous fungi. Moreover, no fungi (and hence yeasts) were recorded in one of them.

Considering the genera representation of filamentous fungi, we confirmed the occurrence of the following genera: *Alternaria*, *Arthrinium*, *Aspergillus* (including previously named as *Eurotium*), *Aureobasidium*, *Cladosporium*, *Mucor*, *Penicillium,* and *Stemphilium*. Isolates of the genus *Penicillium* were recorded with the highest isolation frequency (41%). For comparison, research by authors **Sinacori et al. (2014)**, performed with honey samples of different blossoming, pointed out the occurrence of different fungal species - *Alternaria alternata*, *Aspergillus niger*, *Aspergillus proliferans*, *Aspergillus spelunceus, Chaetomium globosum, Cladosporium cladosporioides, Daldinia concentrica, Emericella discophora, Emericella qinqixianii*, *Penicillium corylophilum*, *Penicillium decumbens*, *Penicillium polonicum*, and *Penicillium echinulatum*, of which *P. corylophilum* and *A. niger* were the most frequent, but in the low count, indicating that the honey is capable of containing multiplication of these fungi. **Rodríguez-Andrade et al. (2019)** have surveyed and evaluated the presence of xerotolerant and xerophilic fungi in a set of honey bee samples collected from across Spain. From 84 samples, a total of 104 fungal strains were isolated. They identified 32 species distributed across 16 genera, most of them belonging to the ascomycetous genera *Aspergillus*, *Bettsia*, *Candida*, *Eremascus*, *Monascus*, *Oidiodendron*, *Penicillium*, *Skoua*, *Talaromyces,* and *Zygosaccharomyces*. As a result of this survey, eight new taxa were proposed. Other fungi rarely found in their study were *Alternaria multiformis*, and the mucoralean *Cunninghamella bertholletiae*, *Mucor plumbeus*, and *Rhizopus oryzae*. These probably represent environmental contaminants. **Martins, Martins and Bernardo (2003)** tested 80 multi-species flower kinds of honey, commercially available in Portugal. They focused on the spores of bacteria and fungi. Yeasts and microscopic filamentous fungi were detected in 88.8% of the samples, identifying 3 filamentous fungus genera - *Aspergillus*, *Penicillium,* and *Mucor* and 2 yeast genera - *Saccharomyces* and *Candida*. *Aspergillus* spp. fungi are ubiquitous and associated with disease in many insects, plants, animals, and man. They are regarded as opportunistic pathogens that require immunocompromised hosts to establish infection.

Microbiological studies have shown a high prevalence of *Aspergillus* spp. in apiaries which occur saprophytically on hive substrates. However, the specific conditions required for pathogenicity to develop remain unknown **(Foley et al., 2014)**. The results of **Foley et al. (2014)** confirm the ubiquity of *Aspergillus* spp. in the apiary environment and highlight their potential to infect both larvae and adult bees unknown. **Bignell (2010)** reported, that food with acidic pH, low humidity, and high concentration of sugars, such as honey, are sources for growth of the fungi *Aspergillus glaucus*. In our study, *Aspergillus* species were isolated from 19% honey samples.

The analysis of filamentous microscopic fungi is now highly emphasized **(Kačániová et al., 2006)**. This is because they can produce mycotoxins that adversely affect human health and reduce the hygienic quality of food. However, the presence of fungi does not imply the presence of mycotoxin; it has necessary ideal conditions such as high water activity, the presence of sugars, and the presence of organic acids capable of reducing pH. Necessary conditions for fungal growth are not always the necessary conditions for the production of mycotoxins **(Barkai-Golan and Paster, 2008)**. Several of the fungi found in honey samples (*Aspergillus* spp. and *Penicillium* spp.) are potential producers of mycotoxins, but this does not mean that the honey may represent a risk to the health of the consumer, because (in general) the production of mycotoxins or the fungal growth is suppressed at water activities lower than 0.70, as is the case of honey (a_w of 0.60 or less) **(Rodríguez-Andrade et al., 2019)**. Despite inappropriate condition found in honey for mycotoxin production, it is important to say that the presence of the fungus can also cause disease in different ways, as induction of allergic responses and infections **(An, 2004)**.

Honey with added fruit

It is generally known that the raw materials must be of excellent quality to produce a good quality product. If poor quality raw materials with certain defective elements are used, these defects will also pass to the final product. High-quality mature honey is considered to be a food with a minimum number of microorganisms and many beneficial effects in terms of human nutrition compared to other foods and no or negligible health risks. In this part of the study, we focused at first on mycological analysis of the raw materials used for the preparation of honey with the addition of fruit. Secondly, mycological analyses of the final products were carried out. Information concerning the counts of isolated fungi **(Figure 2)** is supplemented by information on the water activity of the individual samples **(Figure 3)**, which has a significant impact on their occurrence.

Analysis of the basic raw material - rapeseed pasted honey did not show the presence of microscopic fungi. The water activity of this honey sample was 0.567. **Finola, Lasagno and Marioli (2007)** reported that the low number of filamentous micromycetes in honey is a good indicator of suitable conditions in the apiary environment.

Honey with blueberries

Regarding blueberries, only frozen fruits had a water activity value suitable for the growth of microorganisms (0.928). Yeast had the highest values in the lyophilized raw material. This is also associated with the highest yeast value in honey with lyophilized blueberries. According to **Dobiáš (2004)**, microorganisms are present in lyophilized products in the inactive state, but when added to honey, they may rehydrate and thus become active. Similarly, the highest number of filamentous fungi we detected in the lyophilized blueberries and the honey with lyophilized blueberries. Filamentous micromycetes were not present in dried blueberries and the product thereof. The water activity of the products ranged from 0.524 to 0.592 – optimal water activity in terms of long-term product stability.

The filamentous fungi most commonly identified in blueberries, were zygomycetes *Rhizopus* spp. and *Mucor* spp. The presence of *Rhizopus* spp. was also observed in honey with lyophilized blueberries. Blueberries can be perishable at high moisture content. After blueberry maturation, physiological changes and tissue softening will occur. The fruits used for the manufacture of the products must not be oppressed or rotten, must be free from infested or otherwise damaged parts **(Zambiazi et al., 2016)**.

This product may have significant nutritional properties. **Zambiazi et al. (2016)** investigated the antioxidant activity and bioactive substances in blueberries and honey products with the addition of blueberries. Honey with the addition of blueberries achieved excellent results in this experiment regarding the phytochemical content and antioxidant capacity, due to the presence of both raw materials in this product.

Honey with cranberries

In terms of the highest water activity (0.905) of raw materials, fresh cranberries appear to be the most hazardous. After the addition of fresh cranberries to honey, the water activity of the honey partially increased (0.640). According to **Woodbine (1983)**, osmophilic yeasts could be able to grow in this water activity. However, yeasts were found at relatively low values only in samples of fresh and lyophilized cranberries. They were not even present in honey with lyophilized cranberries, although they were detected in the raw material. The average number of yeasts found in honey with fresh and dried cranberries was in both cases at the limit of detection of 1 log $CFU.g^{-1}$.

The occurrence of filamentous micromycetes in raw materials can be considered as low. According to **Woodbine (1983)**, they can grow in a water activity range of 0.87 – 0.80 and the xerophilic filamentous micromycetes have a water activity range of 0.75 – 0.65. We detected representatives of *Cladosporium* and *Penicillium* genera. Within the products, filamentous fungi were found only in a sample of honey with fresh cranberries, where their number was at the limit of detection. **Tadych et al. (2015)** mentioned, that cranberry fruit is a rich source of bioactive compounds that may function as constitutive or inducible barriers against rot-inducing fungi. It is well known that it naturally contains benzoic acid, and is commonly added as a preservative to many other fruit and berry products. Higher content of benzoic acid guarantees a good shelf life of fresh and processed fruits **(Mateljan, 2017)**.

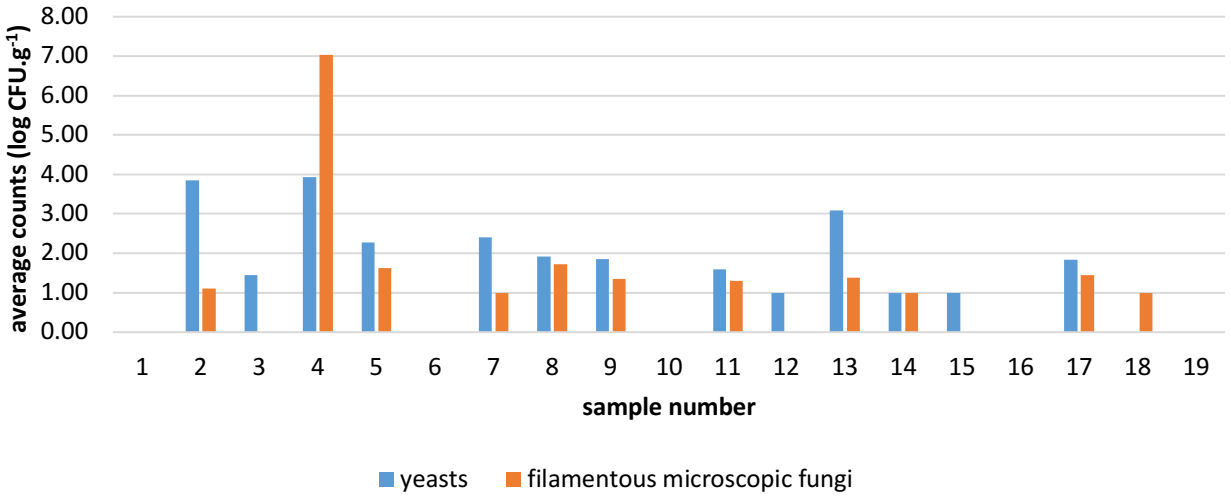

Figure 2 Mean counts of microorganisms detected in raw materials used for the production of honey with additives and in final products.
Note: (1 – honey, 2 – frozen blueberries, 3 – dried blueberries, 4 – lyophilized blueberries, 5 – fresh cranberries, 6 – dried cranberries, 7 – lyophilized cranberries, 8 – frozen red currants, 9 – dried red currants, 10 – lyophilized red currants, 11 – honey with frozen blueberries, 12 – honey with dried blueberries, 13 – honey with lyophilized blueberries, 14 – honey with fresh cranberries, 15 – honey with dried cranberries, 16 – honey with lyophilized cranberries, 17 – honey with frozen red currants, 18 – honey with dried red currants, 19 – honey with lyophilized red currants).

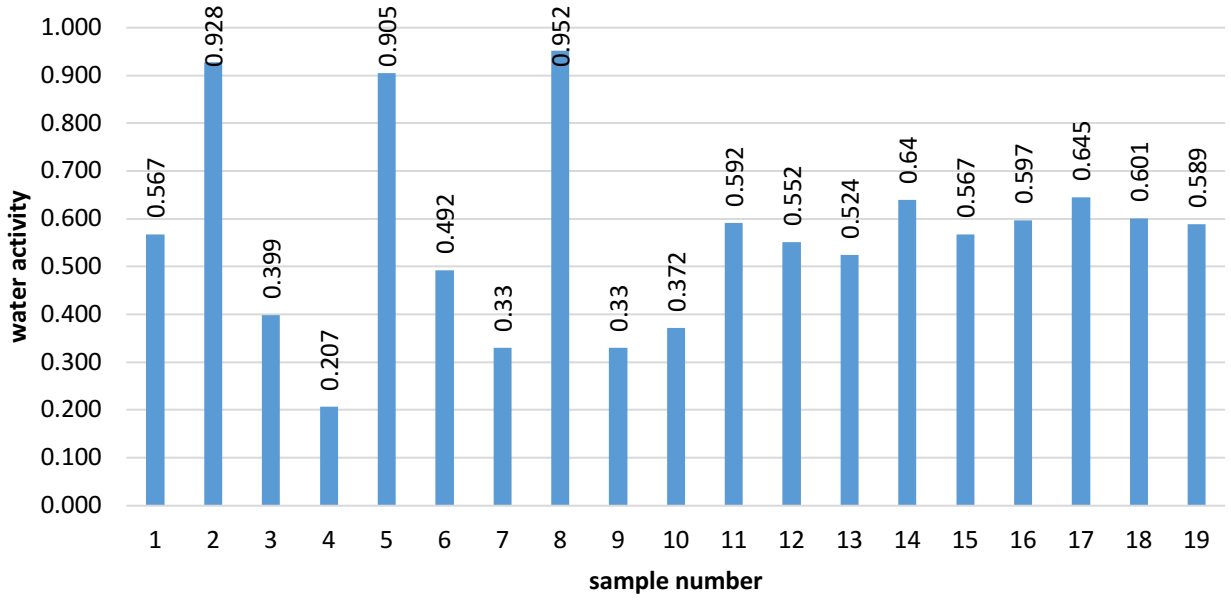

Figure 3 Water activity (a_w) of raw materials used for the production of honey with additives and of final products.
Note: (1 – honey, 2 – frozen blueberries, 3 – dried blueberries, 4 – lyophilized blueberries, 5 – fresh cranberries, 6 – dried cranberries, 7 – lyophilized cranberries, 8 – frozen red currants, 9 – dried red currants, 10 – lyophilized red currants, 11 – honey with frozen blueberries, 12 – honey with dried blueberries, 13 – honey with lyophilized blueberries, 14 – honey with fresh cranberries, 15 – honey with dried cranberries, 16 – honey with lyophilized cranberries, 17 – honey with frozen red currants, 18 – honey with dried red currants, 19 – honey with lyophilized red currants).

Honey with red currants

Red currants are a valuable component of a healthy diet because they are an excellent source of ascorbic acid, anthocyanins, and minerals **(Nour, Trandafir and Ionica, 2011)**. The water activity of frozen red currants was the highest (0.952) of all raw materials used in this study.

Woodbine (1983) reports that sporulating bacteria, gram-negative rods, or some yeasts can multiply in such processed fruits. In both dried and lyophilized currants, the water activity values were considerably lower and the growth of microorganisms would not be possible. The highest number of yeasts and filamentous fungi was recorded in frozen currants. Based on this finding, we assume their occurrence in the composite product as well, which was confirmed. Also, this product had the highest water activity (0.645). However, the yeast and filamentous fungi counts were not high (below 2.00 log CFU.g^{-1}) and some self-regulation is possible over time. The dominant fungi isolated from this raw material were *Cladosporium* spp. Yeasts or filamentous micromycetes did not occur in

lyophilized currants or honey with their addition. Yeasts were also not identified in honey with dried fruit.

CONCLUSION

Honey mycobiota can vary in qualitative and quantitative terms, depending on many factors, such as water activity, or various physico-chemical and biological parameters. For mycological diversity, which can have a significant impact on its quality and safety, it is one of the relatively few investigated commodities. Both yeast and filamentous microscopic fungi can affect honey properties. A total of 27 kinds of honey tested in this study have shown good mycological quality. For yeast occurrence, we can consider our honey samples as safe (except one flower honey sample) because they did not exceed the value 3.00 log CFU.g^{-1}. The average value in positive samples was 1.94 log CFU.g^{-1}. Similarly, the occurrence of filamentous microscopic fungi in honey samples does not present a significant risk. Their numbers did not exceed 2.50 log CFU.g^{-1} and the average number in positive samples was 1.51 log CFU.g^{-1}. Although we have observed the presence of potential mycotoxin producers, they do not have suitable conditions for production.

In the case of good physico-chemical properties of honey and in particular of sufficiently low water activity, microscopic fungi may survive, but they do not multiply and thus spoil the honey. On the other hand, in the production of various honey products, it is very important to have these properties of products under control, as these microorganisms could reproduce, cause unwanted changes and affect its safety. To produce honey with added fruit we have to use raw materials of excellent (microbiological) quality. Deficiencies in honey, blueberries, cranberries, and red berries can lead to the spoiling of the final product. It is also important to choose the appropriate combination and concentration of raw materials. The study showed that cranberries were the most appropriate addition from a mycological point of view. This may also be related to the reported benzoic acid content. In the case of cranberries and red currants, their lyophilized and dried forms were the most suitable. We have not observed the presence of yeasts or filamentous micromycetes in honey with lyophilized cranberries or currants. In the case of honey with blueberries, the dried fruit variant was the most suitable. Based on the water activity of honey with additions, all products appeared to be stable.

The use of honey in products that receive no or limited heat treatment may require additional tests. More information on the source and control of microorganisms in honey is needed to answer the concerns currently facing the industry. Moreover, it would be advisable in further research to monitor the products in storage studies.

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POLLEN DIVERSITY IN HONEY OF THE CZECH REPUBLIC IN THE 2019 SEASON

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ABSTRACT

Honeybees are important pollinators. As a side product of pollination, honeybees produce honey, as a natural sweetener. The source of honey depends on the hive location. In specific conditions honeybees produce monofloral honey, but more common are polyfloral kinds of honey. In this study honey from the Czech Republic in the 2019 season was evaluated by melissopalynology analysis. The common botanical taxa in the Czech Republic were determined and season impact to pollen taxa was compared for dominant pollen taxa. The taxonomic distribution of pollen in Czech honey was stable during the year. The average number of species was 11.52 taxa per sample. The dominant pollen source in Czech honey was the *Brassicaceae* family. The high pollen content in honey was confirmed also in the *Rosacea* family (fruit tree), *Ubelliferacae* family and *Myosotis* genus. During the year the pollen taxa were equally distributed in honey. Seasonal effects were confirmed only in *Salix* genus, *Ubelliferacae* family and *Phacelia* genus. Seasonal effects correspond with the blooming season and honeybee handling in the hive was also confirmed. High variability during the season and hive location was confirmed for other taxa.

Keywords: melissopalynology; botany taxa; pollen; biodiversity; adulteration

INTRODUCTION

The Czech Republic is one of the Central European countries. Its geographical position roughly in the center of the European continent means that the flora in this country includes plant species from the cold north and warm south as well as the oceanic west and continental east. The country is covered by a heterogeneous mosaic of cultural landscapes with arable fields, deciduous, mixed and coniferous forests, meadows, pastures, and human settlements. The dominant type of natural vegetation is a forest. Natural treeless vegetation includes alpine and subalpine grasslands, steep rocky slopes, steppe, peat bogs, and natural water bodies **(Kaplan, 2012)**. The flora includes 148 families of vascular plants, 925 genera, 3754 species and subspecies, and 618 hybrids. Genera with 30 or more species include *Taraxacum* (221 species), *Rubus* (127), *Carex* (85), *Hieracium* (59), *Pilosella* (59), *Veronica* (35), and *Trifolium* (34), four of which include agamospermous species, which accounts for the high diversity. Families richest in species are the *Asteraceae* (666 species), *Rosaceae* (315), *Poaceae* (273), *Fabaceae* (171), *Brassicaceae* (148), *Cyperaceae* (127), *Lamiaceae* (112), *Caryophyllaceae* (108), and *Apiaceae* or *Umbelliferae* (99) (Danihelka, 2013). Due to the human factor activity, the landscape changes and some plant species gradually disappear (Grulich, 2012), while other non-native plants are introduced into the Czech ecosystem **(Pyšek et al., 2012)**. The list of botanical species is extensive. In addition to the diversity and stability of the landscape, some taxa also participate in the honey collection of bees in the Czech Republic. The share of individual plants in the honey collection varies. It all depends on the amount of nectar and pollen produced, which is reflected in the different attractiveness of botanical species for bees. The pollen grains present in honey can be used to determine the botanical origin of it **(Von Der Ohe et al., 2004)**.

Table 1 Number of pollen grains in selected unifloral honey **(Demianowicz, 1964)**.

Type of unifloral honey	The average number of pollen grains / 10 g of honey,
Myosotis silvatica	147,456,000
Brassica napus	72,000
Taraxacum officinale	18,000
Malus domestica	18,000
Robinia pseudoacacia	1,125
Phacelia tanacaetifolia	72,000
Tilia sp.	2,250

Table 2 Referee values for morphological and spectral characteristics.

Taxon	Length	SD	Width	SD	Length / Width	L*a*b*
Brassica sp.	29.22	1.15	20.36	0.64	1.44	94.30;-5.09;21.62
Corylus sp.	27.62	2.04	19.31	1.75	1.43	98.67;-5.70;14.66
Artemisia sp.	23.64	1.95	16.26	0.93	1.45	97.27;-3.96;11.18
Alnus sp.	38.90	19.31	26.86	13.42	1.45	97.04;-5.20;16.05
Fruit tree	42.47	8.68	24.17	4.72	1.76	97.61;-2.09;6.21
Robinia sp.	33.58	2.93	21.42	3.36	1.57	98.02;-3.12;9.65
Rubus sp.	23.64	2.51	14.89	1.86	1.59	97.40;-3.96;11.35
Salix, Salicaceae	18.99	0.8	12.78	0.82	1.49	96.73;-5.02;16.07
Bellis sp.	31.53	1.05	19.66	1.35	1.60	91.94;-4.37;18.91
Acer sp.	41.66	2.06	22.52	2.41	1.85	93.76;-1.27;16.43
Helianthus sp.	33.05	4.03	26.33	3.56	1.26	96.38;-3.59;17.32
Fagus sp.	33.96	6.15	23.43	4.22	1.45	99.27;-3.24;6.37
Trifolium sp.	25.73	4.47	15.52	2.85	1.66	96.65;-3.58;12.96
Tilia sp.	20.1	3.95	14.18	2.87	1.42	96.35;-2.72;10.78
Phacelia sp.	20.76	1.58	14.38	1.23	1.44	95.58;-4.22;13.89
Rhamnus sp.	59.46	5.29	24.15	2.26	2.46	98.30;-3.44;20.23
Umbelliferae	23.67	2.5	13.75	1.34	1.72	96.96;-3.64;11.75
Achillea sp.	33.49	6.3	20.11	2.15	1.67	97.43;-3.22;11.35
Vicia sp.	44.1	3.86	30.29	5.3	1.46	91.74;-2.39;24.78
Taraxacum sp.	34.71	2.05	21.95	2.68	1.58	94.28;-6.39;39.21
Myosotis sp.	14.56	2.86	7.29	1.3	2.00	95.07;-3.13;10.04

However, when determining the botanical origin, it is necessary to consider the unlike the production of pollen by botanical taxa, which was experimentally verified in the study by Deamianowics 1964 **(Table 1)**.

At present, the pollen profile typical for Czech honey has not been described. Such data can be used not only to expand knowledge but can also play a crucial role in preventing honey adulteration. The origin of honey can be proven under certain conditions based on the pollen profile that represents the area where the honey comes from **(Aronne and de Micco, 2010; Soares et al., 2017)**. Some countries have their pollen profile of honey described. Monofloral honey is the ones characterized most commonly **(Persano Oddo and Piro, 2004; Oddo et al., 1995; Persano Oddo et al., 2004; Feás et al., 2010; Karabagias et al., 2020)**. Fewer studies have focused on the pollen profile of polyfloral honey **(Kuś et al., 2018; Čeksteryte, Kurtinaitiene, and Balžekas, 2013; Kale Sniderman et al., 2018; Puusepp and Koff, 2014; Jones and Bryant, 2014)**. For the characterization of monofloral honey, in particular, the number of pollen grains of the species as well as the amount of accompanying pollen grains must be taken into account.

This study aims to bring new knowledge about the pollen profile of Czech honey in 2019.

Scientific hypothesis

The pollen profile of honey is closely dependent on the area of collection of nectariferous and nectarless plants around the hive. Pollen profile variation during the year was verified in this study.

MATERIAL AND METHODOLOGY

Sampling Collection

The experimental material was collected from individual colonies of western honeybee *Apis mellifera carnica*. One or two sealed honeycombs from each colony were extracted using a common hand extractor. All samples were from the 2019 season. The samples were collected from May to August and classified into four groups depending on the month of their collection. The 163 samples were evaluated from 130 different areas of the Czech Republic with various geographical profiles and botanical origins. Pollen profile in posterior months is influenced by the natural handling of honey in the hive. The honey handling can cause temporal and also positional shifts according to beekeeping practice **(Vorwohl, 1972)**.

Pollen Analysis

Honey samples were prepared following the guidelines of the International Commission of Bee Botany published by von der Ohe **(Von Der Ohe et al., 2004)**. Glycerol-gelatine preparations were made in duplicate for each honey. The pollen spectrum was evaluated by Nikon Eclipse Ci-L (Nikon, JPN). The slide was automatically scanned by the motorized stage with the focus motor of Proscan III (Prior, USA). Images were captured in stag files using DFK 23U274 camera (Imaging Source, GER). The position was chosen randomly. The magnifications used were 100x and 400x.

The pollen spectrum was classified according to Stawiarich **(Stawiarz and Wróblewska, 2010)** into four groups >45% dominant, 16 – 45% secondary, 3 – 15%

important minor and <3% minor pollen. Pollen discrimination was performed according to Moar **(Moar, 1985)**. At least 300 pollen grains were counted in each preparation, where pollens were identified according to melissopalynology atlas **(El-Labban, 2020)** to the most possible exact taxon – species, genus, type of structure or family in classes >3%. Pollen not clearly identified by the evaluator was evaluated by morphometric and spectral characteristics obtained from image analysis. Referee values are shown in **Table 2**.

Statistical analysis

The data were processed statistically using the 2014.5.03 XLSTAT software (Addinsoft, USA). The normality test confirmed the not normal distribution of the data. A nonparametric Kruskal-Wallis test was used to compare the pollen profile in months and the diversity of pollen taxa during the year.

RESULTS AND DISCUSSION

Honey samples were collected during the 2019 beekeeping season, specifically from 18 May to 16 August 2019. The period was selected to include both spring and summer honey phases. Such a long period of the collection of samples enables covering botanical taxa of nectar-producing and pollen-producing plants involved in honey production in the Czech Republic. 163 honey samples were taken. Of these, 30 honey samples could be characterized as monofloral concerning their pollen spectrum. These were namely (*Brassica sp.* 8; *Prunus sp.*, *Pyrus sp.* 18; *Tilia sp.* 4). It is generally stated that if there is more than 45% of pollen grains of one species in certain honey, the honey can be described as single-species or monofloral. However, this rule does not apply to all botanical taxa. Some plants differ in their pollen-producing capacity, both in the high content of pollen grains and, conversely, due to the low content of pollen grains **(Demianowicz, 1964)**. The rule that several botanical species contribute to the composition of nectar and pollen content even in monofloral honey also applies **(Louveaux, Maurizio, and Vorwohl, 1970)**. Of the analyzed samples, 133 kinds of honey were polyfloral. The representation of individual taxa in Czech honey is summarized in **Figure 1** and these taxa are divided into four groups, namely <3% minor pollen, 3 – 15% important minor 16 – 45% secondary and >45% dominant.

The wide pollen spectrum in Czech honey is characteristic of Mediterranean areas with a high degree of urbanization and agricultural activity. Even about the large sown areas of crops in the Czech Republic, Czech honey retains in most cases the character of polyfloral honey. Differences in diversity by hive location were described in a French study **(Odoux et al., 2012)**. Here, the authors confirmed a reduction in the diversity of pollen taxa in agricultural areas. For the Czech Republic, however, there has been no similar study yet that would allow a comparison of the change in the number of pollen taxa in agricultural areas. The results of the study showed an average of 11.63 pollen taxa in pollen with a proportion of >3% occurrence of pollen grains in honey **(Figure 2)**.

Another monitored parameter was the differences in the number of pollen taxa throughout the honey season. Taxa with a frequency of presence greater than 3% pollen grains were observed **(Figure 2)**. **Figure 2** shows the number of pollen taxa during the year per sample. A shorter distance between curve points means a shorter period between sampling in a given period. The time interval between sampling at the beginning and end of the season is longer. Both the climatic conditions and the source of nectar at the given habitats affect the time interval.

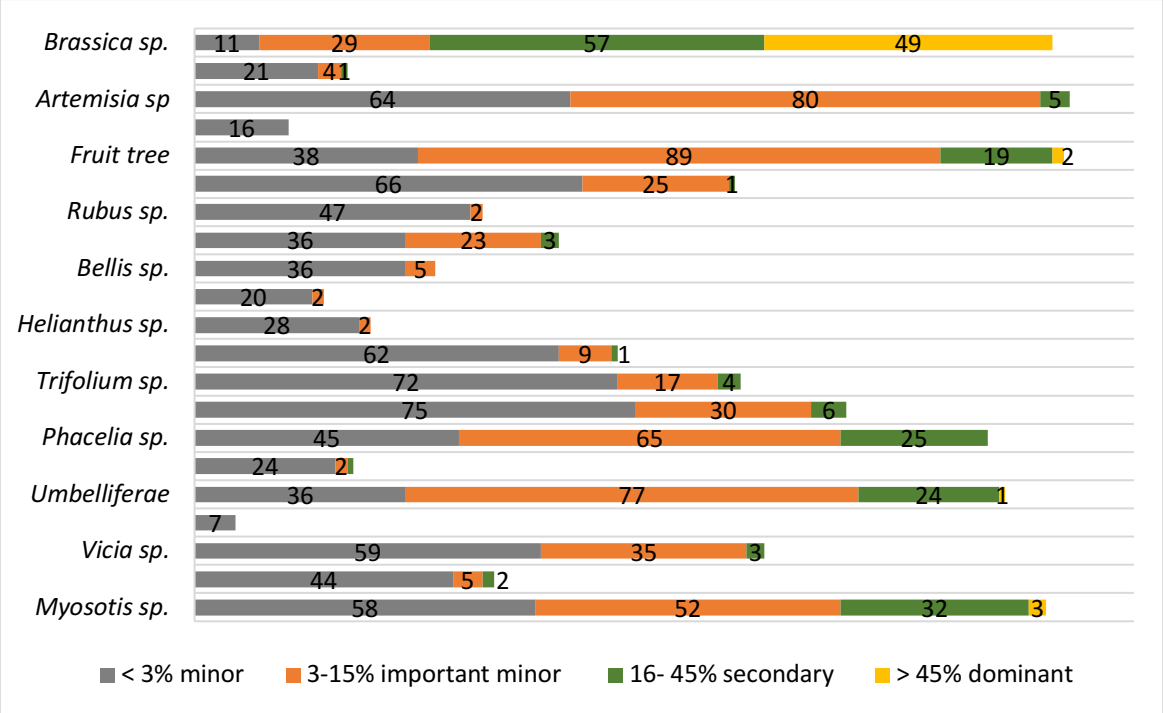

Figure 1 Absolute frequency of major pollen taxa in Czech honey in 2019.

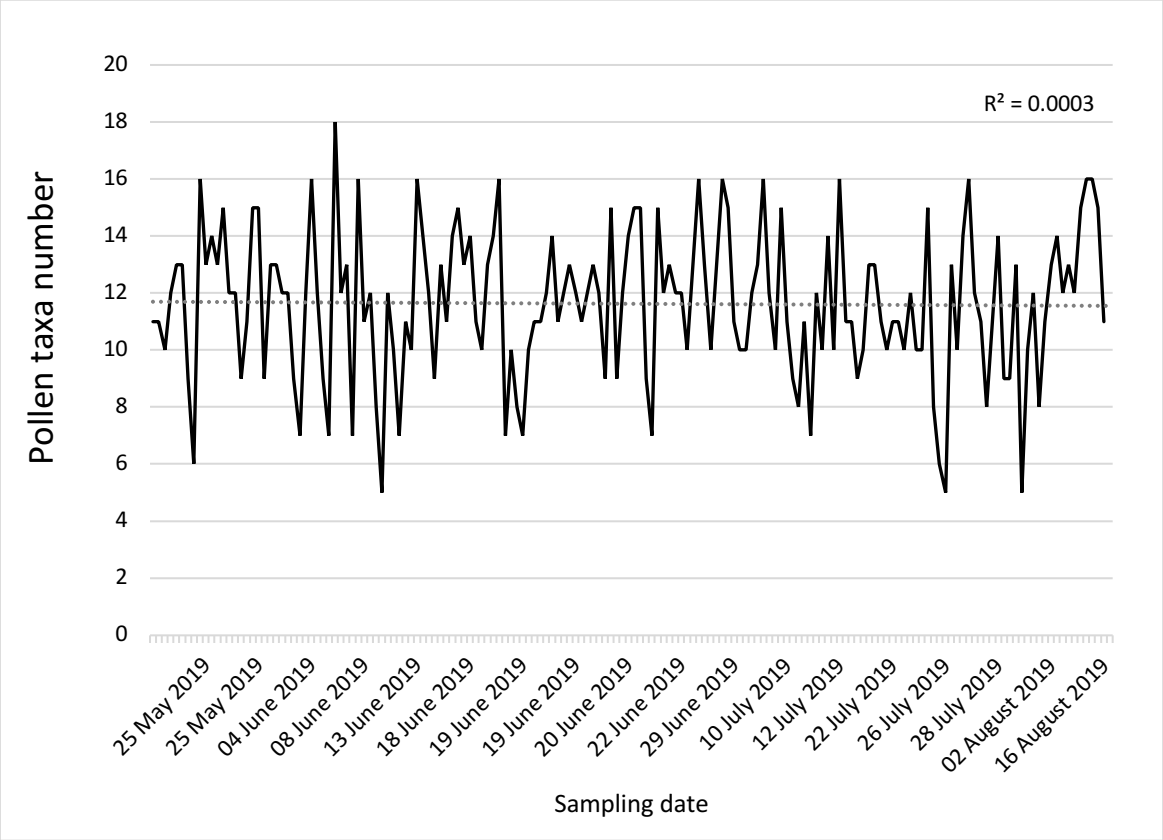

Figure 2 Taxa diversity in 2019.

For the Czech Republic, no statistically significant differences were recorded between the date of honey extraction and the diversity of pollen taxa (p >0.05). This result is also confirmed by **Figure 2** which does not confirm the change in individual months. The differences in the number of pollen taxa correspond to specific habitats, not to the time of the year. The average diversity in the spring was 12 taxa in May and 11.62 taxa in June. In summer it was 10.83 taxa in July and 11.63 taxa in August. The result is in agreement with the study **(Avni et al., 2014)** where, using chemical analysis, the authors showed that the amount of pollen in the pollen collection does not differ during the year, but is dependent on the habitat. Primarily in the spring period, pollen is responsible for the rapid development of bee colonies **(Odoux et al., 2012)** and subsequently contributes to honey yields. In the conditions of the Czech Republic, spring pollen includes primarily pollen of fruit trees. Fruit tree pollen is considered a very good source of protein for bee colonies **(Roulston and Cane, 2000)**. Due to its nutritional importance, fruit tree pollen plays an important role in bee nutrition and is associated with a high preference for bees. Even concerning lower pollen-producing capacity **(Table 1)** than in *Brassica sp.,* the pollen of fruit trees was represented in honey on average in the amount of 8.82%, in most cases, it was an important minor **(Figure 1)**. Differences between the compared months were not confirmed, but the proportion of pollen grains of fruit trees in later months, as well as other pollen sources, confirm the pollen cycle in honey within a year **(Figure 3** and **Figure 4)**. The occurrence of pollen of spring botanical species in later months is mainly due to the growth of the brood and the transport of pollen both on bees as well as by bees to their honeycombs. The influence of the pollen profile on the distance from the brood, but also the humidity in the hives was confirmed by Spanish authors (**Da Fernandez and Ortiz, 1994)**. Fruit trees include several genera and even more cultivars, but concerning their close relationship, the morphology of the pollen grain is similar, although there are differences between the pollen grains, especially in the color of the pollen **(Pospiech et al., 2019)**. For melissopalynological purposes, they are often taken as one group **(Stawiarz and Wróblewska, 2010)**. The fact that the honeybees' visits to flowers are not influenced by an exclusive species preference also makes it difficult to determine the exact species. On the other hand, the species non-specificity of the honeybee is used in orchards, where the bee is a significant pollinator **(Cunningham et al., 2016)**. Single-species honey of fruit trees is not widespread. Their occurrence has been described, for example, in Bulgaria. Due to their sensory closeness, they may be confused with other spring honey, primarily with black locust honey **(Atanassova, Yurukova, and Lazarova, 2012)**. A high proportion of pollen from *Brassicaceae* and fruit trees was also found in Polish polyfloral honey, see **Table 3** (**Stawiarz and Wróblewska, 2010)**.

Other important sources of pollen in spring honey include pollen of the *Brassicaceae* family (especially *Brassica sp.*), dandelion, hazel, and black locust **(Figure 3)**. High *Brassica sp*. pollen content is recorded primarily in honeybee colonies near the agricultural areas with these plants **(Danner et al., 2016)**. In these areas, honey can also reach the character of monofloral honey.

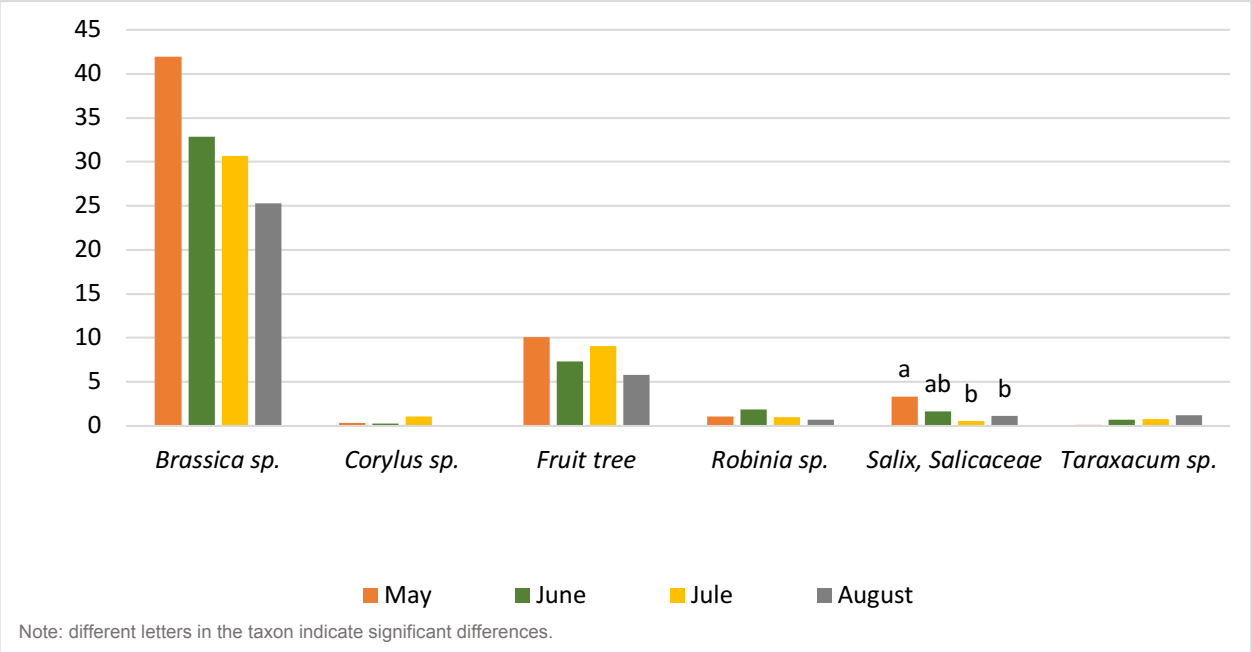

Figure 3 Average occurrence of pollen of spring botanical taxa.

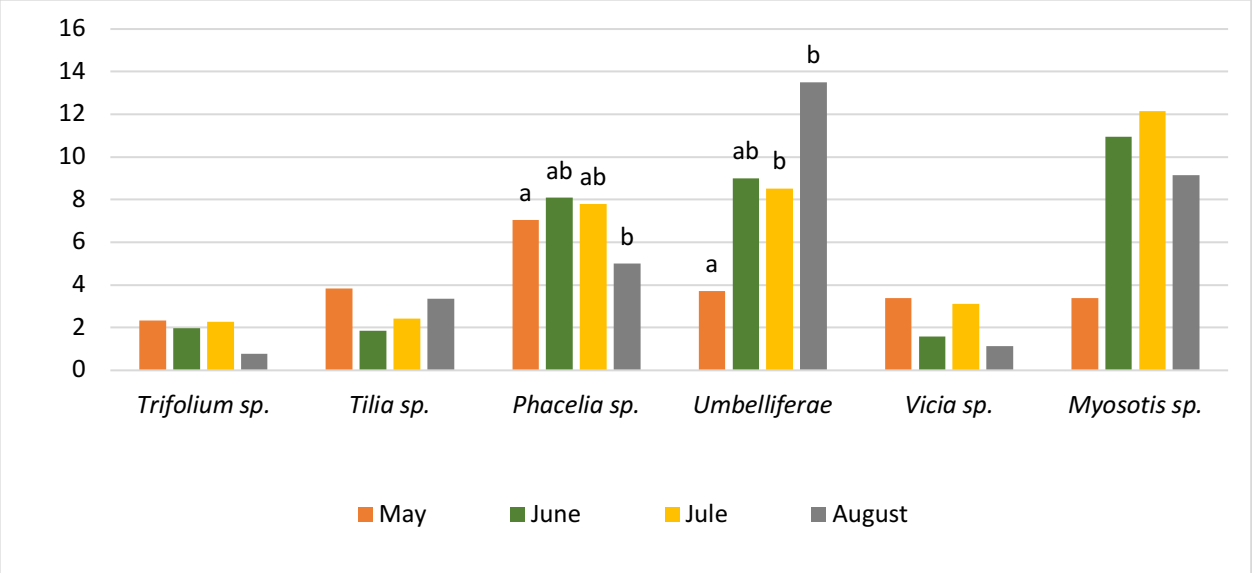

Figure 4 Average occurrence of pollen of summer botanical taxa.

Due to the high pollen-producing capacity, honey with a rapeseed pollen content of more than 80% can be considered monofloral rapeseed honey **(El-Labban, 2020)**. Of the honey analyzed in our study, 8 honey samples would meet this definition. However, high content of rapeseed pollen grains was also recorded in polyfloral honey, with more than 16% of pollen grains in 106 honey samples.

The high content of pollen grains of *Brassica sp.* is mainly due to its pollen-producing capacity **(Table 1)**. It was experimentally verified that it belongs among the most pollen-producing plants involved in spring honey collection **(Demianowicz, 1964)**. We found the average proportion of rapeseed pollen at 35.43%, in most cases it was the secondary pollen **(Figure 1)**. The high proportion of rapeseed pollen was also confirmed in Polish and Estonian honey **(Stawiarz and Wróblewska, 2010; Puusepp and Koff, 2014)**.

Salicaceae pollen is considered to be an important source of protein needed for honeybee colony development, although willow also provides some nectar. Honeybees mainly search for male flowers for their source of pollen grains **(Dötterl et al., 2014)**. The average proportion of *Salicaceae* pollen was 4.2% and in most cases, it was minor pollen. Our results showed statistically significant differences in the amount of *Salicaceae* pollen grains in May-June honey, compared to July-August honey **(Figure 3)**. The august increase shows also the temporal shift of pollen caused by handling in the hive as confirmed previously by **(Da Fernandez and Ortiz, 1994)** and **(Vorwohl, 1972)** for *Myosotis* pollen. Although monofloral *Salicaceae* honey is not described in the Czech Republic, monofloral *Salicaceae* honey can be found in Croatia, Spain, Lithuania, and New Zealand **(Jerković and Marijanović, 2010)**.

Table 3 Percentage of pollen in polyfloral honeys of selected botanical taxa.

	Czech Republic*			Portugal (Sousa et al., 2014; Feas et al., 2012)			Poland (Stawiarz and Wróblewska, 2010)			Estonia (Puusepp and Koff, 2014)		
Taxon	x̄	Min.	Max.	x̄	Min.	Max.	x̄	Min.	Max.	x̄	Min.	Max.
Robinia sp.	2.43	0.17	16.01	16.00	13.00	22.00	28.13	3.00	27.70	8.50	8.50	8.50
Fruit tree	8.88	0.3	65.29	23.36	1.00	80.00	30.40	4.00	21.10	14.22	0.80	56.90
Rhubus sp.	1.38	0.17	12.25	4.00	2.10	5.60	-	-	-	4.91	0.60	16.70
Trifolium sp.	3.47	0.15	33.62	13.30	4.40	45.60	18.10	-	-	11.90	0.40	71.40
Brassica sp.	35.43	0.46	94.12	12.00	12.00	12.00	33.26	16.2	59.60	9.81	2.00	31.30
Taraxacum sp.	2.23	0.15	16.41	-	-	-	40.20	40.2	40.20	4.23	0.30	13.60
Phacelia sp.	8.68	0.17	40.8	-	-	-	26.73	3.00	18.70	12.15	4.80	19.50
Salixaceae	4.02	0.19	19.29	-	-	-	30.57	16.3	44.10	11.30	2.73	29.00
Tilia sp.	3.52	0.17	25.38	-	-	-	-	-	-	44.74	1.30	79.03
Umbelliferae	10.03	0.29	65.23	-	-	-	-	-	-	15.80	0.50	82.80

Note: * honeys with a pollen content higher than 0 are included.

Robinia pseudoacacia pollen was confirmed in honey in all monitored months. Its average representation was 2.43%. In most cases, it had a minor representation, which corresponds to the findings by other authors **(Stawiarz and Wróblewska, 2010; Čeksteryte, Kurtinaitiene, and Balžekas, 2013)**. In June, the content of *Robinia pseudoacacia* pollen was the highest and one sample contained 16.01% of this pollen. Although some authors state that >15% of *Robinia pseudoacacia* pollen indicates monofloral honey **(Oddo et al., 1995)**, more authors are inclined to the 20% limit **(El-Labban, 2020)**. The reason for the different minimum limit of pollen grains in single-species black locust honey is mainly the low pollen-producing capacity **(Table 1)** and the high nectar-producing capacity of black locust.

Monofloral dandelion honey is also characterized by a low content of pollen grains, which is usually in the range of 5 – 15% in monofloral honey as well **(Jerković et al., 2015)**. In the pollen profile of these honey, dandelion pollen is often lower than the associated species, such as *Salix* or *Cruciferae* **(Persano Oddo and Piro, 2004)**. The average proportion of dandelion pollen was 3.62% and in most samples, it was minor pollen **(Figure 1)**. According to the pollen profile, one sample would meet the 15% condition. The accompanying pollen was *Myosotis sp.* pollen (23.33%), *Phacelia sp.* pollen (11.03%,) and fruit tree pollen (10.26%). Various concomitant pollens (*Salix sp.* 33% and *Brassica sp.* 16%) were also confirmed in the study **(Jerković et al., 2015)**. The lowest proportion offspring-flowering trees in Czech honey was represented by hazel pollen, **Figure 3**. The average amount reached 2.96% and in most cases,s it was minor pollen. Hazel is one of the spring pollen-producing plants. The reason for its low incidence might be climatic conditions or the use of pollen exclusively for the development of honeybee colonies **(Odoux et al., 2012)**. The presence of hazel pollen in honey has also been confirmed in Estonia **(Puusepp and Koff, 2014)**, Germany, Australia **(Bibi, Husain, and Naseem, 2008)**, and Lithuania **(Čeksteryte, Kurtinaitiene, and Balžekas, 2013)**.

The representation of the main summer pollen taxa is summarized in **Figure 4**. *Myosotis sp.* pollen had the highest proportion in honey (10.99%), but even so, it had a minor or important minor representation in most samples **(Figure 1)**. Forget-me-not is one of the plants with the highest pollen-producing capacity **(Table 1)** and for a honey to be classified as monofloral honey, the forget-me-not pollen content must be more than 90% **(van der Ham, Kaas, and Kerkvliet, 1999)**. However, monofloral forget-me-not honey is rare in Europe **(Persano Oddo et al., 2004)**. The occurrence of *Myosotis sp* pollen in honey has been confirmed by several authors, with a varying frequency and amount of this pollen in honey **(Stawiarz and Wróblewska, 2010; Gençay Çelemli et al., 2017; Downey et al., 2005)**. The second most abundant pollen was pollen of the *Umbelliferae* family (10.03%), similarly to *Myosotis* pollen, and in this case, the amount of pollen in honey varies, as reported by individual authors. Some authors consider it minor pollen **(Lieux, 1981)**, some consider it dominant pollen **(Marco et al., 2012)**. In Europe, some species of this family are also found in single-species honey (*Daucus Carota, Coriandrum sativum*) **(Persano Oddo et al., 2004)**. The percentage of pollen of the *Umbelliferae* family in the May-June period differed significantly from the July-August period. The differences are due to the blooming period of this family, which is mostly summer and autumn, primarily for taxa important from the beekeeping perspective **(Abou-Shaara, 2015)**.

The *Phacelia sp.* is an agricultural crop with a short growing season well-known to the beekeepers. It can therefore be used as a source of nectar and pollen **(Sprague et al., 2016)** by beekeepers themselves, or it is used in intensive agriculture for green manure **(Titov and Mamonov, 2013)**. *Phacelia sp.* pollen was confirmed in honey from the Czech Republic and the average content in honey was (8.68%), but in most cases, it had a minor representation **(Figure 1)**. The *Phacelia sp.* pollen occurred mostly in honey from the May-July period, in August honey this pollen was represented less. A statistical difference was demonstrated between May and July. Due to the high pollen-producing capacity, honey with a *Phacelia* pollen

content >90 % is considered monofloral *Phacelia* honey **(van der Ham, Kaas, and Kerkvliet, 1999)**. A more recent Polish study also admits a lower proportion of pollen grains (from 68%) in the case of corresponding physico-chemical and sensory parameters **(Kuś et al., 2018)**.

The most well-known summer nectar-producing tree is considered to be the linden (*Tilia sp.*). Linden has a high nectar content but a low pollen content. Therefore, a low proportion of pollen grains (>20%) is permissible for monofloral linden honey **(van der Ham, Kaas, and Kerkvliet, 1999)**. The average content of pollen grains in the Czech Republic was 3.52% and in most honey, it represented minor pollen in honey. This finding is in line with Polish and Bulgarian polyfloral honey **(Stawiarz and Wróblewska, 2010; Dobre et al., 2013)**. In three honey samples, the pollen content was higher than 20%, which may indicate monofloral honey. The secondary pollen in these samples was rapeseed pollen (1.21 – 19.86%), *Phacelia sp*. pollen (15.05 – 21.75%), and *Umbelliferae* pollen (2.69 – 18.88%).

As reported by several authors **(Lieux, 1981; Kale Sniderman et al., 2018)**, *Vicia sp*. pollen is an important taxon in polyfloral honey, however, according to the study **(Stawiarz and Wróblewska, 2010)**, it is considered a minority representative. In the Czech Republic, its average content of pollen grains was 3.62% and in most cases, it was minor pollen (<3%) **(Figure 1)**. *Trifolium sp.* pollen was represented in Czech honey on average in the amount of 3.47%. It was minor pollen **(Figure 1)** in most honey. The percentage found is lower than described in other countries **(Jones and Bryant, 2014; Stawiarz and Wróblewska, 2010)**. Varying content of pollen grains of Trifolium sp., however, was also observed in various localities in Lithuania, wherein some localities the content of Trifolium sp. pollen was even lower **(Čeksteryte, Kurtinaitiene, and Balžekas, 2013)**.

A comparison of the pollen profile of the Czech Republic in 2019 with foreign studies is summarized in **Table 3**. The selection of taxa is limited concerning the availability of information on the pollen profile of honey from abroad. Botanical taxa for which a comparative study is not available are excluded from the table.

CONCLUSION

In most cases, Czech honey has the nature of polyfloral honey. In 2019, the predominantly represented pollen was of *Brassica sp*. Fruit tree, *Umbelliferae*, and *Myosotis sp.* had a higher percentage as well. The amounts of dominant pollen taxa in Czech honey do not differ significantly during the year. Confirmed botanical taxa were present in honey in all monitored months. Differences between months were confirmed only for pollens of *Salixaceae*, *Umbelliferae* family, *Phacelia sp.* and they are in accordance with the blooming time of these botanical species. The occurrence of pollen taxa in the months out of the main blooming season is caused by physiological handling in the hive, which results in the transfer of honey and pollen cells in the honey flow season. The bee handling management of honey and pollen is also affected by climate conditions and the availability of pollen and nectar sources each year. For this reason, the study will be extended to the following years in order to confirm or possibly exclude the conclusions found in the 2019 season.

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VITAMIN C AND NITRATES CONTENTS IN FRUIT AND VEGETABLES FROM FARMERS' MARKETS AND SUPERMARKETS

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ABSTRACT

Fruits and vegetables are the best food sources of vitamin C. However, fruits and vegetables can be also sources of potentially harmful substances to the human body, nitrates being one of these. The aim of this study was to compare vitamin C and nitrates contents in selected fruits and vegetables from supermarkets and local farmers' markets. Samples of plums, strawberries, apples, spinach, red peppers and tomatoes were chosen for analysis. Content of vitamin C and nitrates was analyzed by HPLC/DAD. The hypothesis was that local market fruits and vegetables contain more vitamin C and fewer nitrates than samples bought in supermarkets. Laboratory analyses showed that there were differences in vitamin C in the case of strawberries, tomatoes and red peppers. The highest level of ascorbic acid was in red pepper samples (141 $mg.100g^{-1}$). In the case of fruit, the highest content was in strawberries (70 $mg.100g^{-1}$). As far as nitrates content is concerned, in three cases out of six, the fruit and vegetables we tested from farmers' markets contained lower concentrations of nitrates than those purchased at supermarkets and the hypothesis was accepted in these cases. There was no significant difference between the nitrate content of the local market and supermarket strawberries and red peppers. Tomatoes had significantly higher nitrate content when purchased at farmers' markets than at supermarkets. Leafy vegetables are considered to be the major source of nitrates, and this was confirmed by this study. The highest content of nitrates was in the spinach sample (2969 $mg.kg^{-1}$). Among all fruit samples, strawberries had the highest nitrates levels (maximum 131 $mg.kg^{-1}$). The results of this work showed that the content of ascorbic acid and nitrates differs significantly depending on the type of fruit or vegetables.

Keywords: ascorbic acid; nitrate; fruit; vegetable; farmers' market

INTRODUCTION

Fruit and vegetables are an important part of human nutrition and their adequate daily consumption can help prevent serious diseases **(WHO, 2003)**. The **WHO (2004)** recommended at least 400 g of fruit and vegetables daily to prevent chronic diseases such as heart and cancer, type 2 diabetes mellitus and obesity, as well as to prevent and reduce micronutrient deficiencies, particularly in less developed countries **(WHO, 2003)**.

Vitamin C is the most important vitamin in fruits and vegetables. Except for humans and other primates, most phylogenetically lower animals can synthesize this vitamin **(Rekha et al., 2012)**. More than 90% of vitamin C in the human diet comes from fruit and vegetables. The highest amounts of this vitamin occur in blackcurrants, citrus fruits, spinach, tomatoes, red peppers **(Lewin, 1976; Lee and Kader, 2000; Saxholt et al., 2008; Rekha et al., 2012)**.

Vitamin C improves immune system function, acts as an antioxidant, and reacts with oxygen and other molecules **(Rekha et al., 2012; Soni et al., 2017)**. According to **EFSA (2017)**, the reference intake of vitamin C for a healthy adult should be 90 mg per day. Vitamin C content in vegetables and fruits depends on several factors, such as variety, habitat, growing year, storage and processing **(Mařáková, 2008; Matějková and Petříková, 2010; Oyetade et al., 2012; Combs, 2017)**.

Vitamin C is a very unstable compound. It is rapidly oxidized with oxidizing agents, especially iron, copper, various enzymes (e.g. ascorbase, peroxidase, cytochrome oxidase) and air/oxygen **(Jeney-Nagymate and Fodor, 2008)**. Moreover, the stability of vitamin C decreases with increasing temperature, pH **(Jeney-Nagymate and Fodor, 2008)** and light access **(Nováková, Solich and Solichová, 2008)**. During processing, the stability of vitamin C is higher in fruit than in vegetables due to their lower pH **(Mařáková, 2008)**.

Nitrates are created in fruits and vegetables mainly via the nitrification of ammonia nitrogen. They play an important role in plant nutrition, growth and development. Higher nitrates concentrations accumulate in leaves, while lower

concentrations are present in roots, tubers, seeds and fruits. For this reason, leafy vegetables (spinach, lettuce, etc.) are the most important source of nitrates and may contain more than 2000 $mg.kg^{-1}$ **(Maynard et al., 1976; Santamaria, 2006)**.

Nitrates themselves are relatively nontoxic, but their metabolites (nitroso compounds), which could be formed in the human body, can cause several health problems **(Santamaria, 2006)**. Consumption of vegetables with high nitrate content may increase the risk of methemoglobinemia or gastrointestinal cancer **(Du, Zhang and Lin, 2007)**. **Bevc et al. (2012)** reported an association between nitrates and cancer of the bladder, ovary, stomach and liver. According to **EFSA (2008)**, the acceptable daily intake for nitrates (ADI) is 3.7 mg per kg of body weight per day. **Temme et al. (2011)** reported a real average daily nitrate intake of 1.38 mg per kg of body weight per day. The main sources of nitrates are vegetables (mainly lettuce) and drinking water. Due to the undesirable effect of nitrates on human health, their content in certain kinds of vegetables is regulated by **Commission Regulation (EC) No 1881/2006**.

On the other hand, intake of small amounts of nitrates can also have a positive effect on the gastrointestinal tract **(Duncan et al., 1999)** and the cardiovascular system **(Webb et al., 2008; Sobko et al., 2010; Lundberg et al., 2011)**. The negative effect of nitrates on human health can be significantly limited by the simultaneous presence of vitamin C **(Kopec, 2010; Shehata, 2010)**.

The accumulation of nitrates in fruit and vegetables depends on many factors - mainly their type or genotype, variety, maturity, climatic conditions (light intensity, air temperature and carbon dioxide concentration), agronomic factors (e.g. timing and form of nitrogen application), soil type and harvest time **(Sorensen, Johansen, and Poulsen, 1994; Colla et al., 2018)** and also by heat treatment (i.e., frying, baking, cooking), storage or preservation **(Prasad and Chetty, 2008; Temme et al., 2011)**.

Farmers' markets are a form of selling agricultural and food goods to the general public, with the aim of a) supporting small and medium-sized agricultural growers, breeders and food producers; b) supplying citizens with fresh agricultural crops and food of mainly national and regional origin; c) creating new social space which, in addition to the sale of agricultural goods, serves to get people together and to bring the urban population closer to the agricultural season and natural cycles; d) revitalizing selected urban areas and improving their ambience. Farmers' markets usually take place in the open air. Only seasonal and local goods should be sold at farmers' markets **(Bohatec, 2011; Sedláček, 2015)**.

The most common products at farmers' markets include fruit and vegetables **(Brůčková, 2012)**. Unlike organic food, there is no legal or generally accepted definition for local food. Many authors describe it as "local food" such foods that were produced within 100 km of the consumer. Other authors argue that local foods are those produced in a certain region or country.

Another positive factor for buying local food is its freshness. At farmer's markets, we find fresh fruits and vegetables that were picked just before the sale, while in supermarkets we can come across food that has traveled to the counters for several days and matured on the way. This also relates to the carbon footprint; the production of local food is significantly more energy-efficient and thus more environmentally friendly **(Brůčková, 2012; Kukla 2012)**.

For most consumers, fruits and vegetables that come from farmers' markets are considered healthier than usual "conventional" supermarket products. However, it is not clear whether these farm products contain more nutrients and/or less harmful substances **(Wunderlich et al., 2009)**. Therefore, this work aimed to compare the content of vitamin C and nitrate in selected fruit and vegetables purchased at farmers' markets and in supermarkets.

Scientific hypothesis

Fruits and vegetables from farmers' markets will have a higher vitamin C content and a lower nitrate content than those from supermarkets.

MATERIAL AND METHODOLOGY

Vitamin C and nitrates levels were monitored in three different kinds of fruit (apples, plums and strawberries) and three different kinds of vegetables (spinach, red peppers and tomatoes). The Jonagold apple variety was used for analysis. For other analyzed fruit and vegetables the variety was not declared by supermarkets and farmer´s markets. Fruit and vegetables (except spinach) were purchased in the period September-November (spinach in the period October-November) in four different supermarkets and four different farmers' markets in the same locality. The same kind of fruit or vegetables were always bought at the same time in the supermarket and the farmers' market.

Samples preparation

All fruit and vegetable samples were cleaned before analysis. Parts that are not consumed were removed. The edible parts were homogenized for 1 minute in a kitchen blender (Zepter, Italy). In the case of ascorbic acid determination, 2.5 g of the sample plus 15 mL of 3% metaphosphoric acid (Honeywell, Germany) were mixed. The sample was filtered through filter paper to a 25 mL volumetric flask, filled up to the mark with the extraction solvent, filtered through a PTFE membrane 0.45 µm filter, and directly injected into the HPLC 20 µL Rheodyne 7725i loop (Rheodyne, USA).

In the case of nitrates determination, 5 g of homogenized sample was placed into a 150 mL beaker with 60 mL of demineralized water and left in an ultrasonic bath for 10 minutes. After that, it was filtered through filter paper to a 100 mL volumetric flask, filled up to the mark with extraction solvent, filtered through a membrane filter, and injected into the HPLC system as mentioned above. Each sample was measured in triplicate.

Instrumental analysis

A high-performance liquid chromatographic (HPLC) method was used to determine ascorbic acid and nitrates contents. The HPLC system (P680 HPLC pump, Thermostatted Column Compartment TCC–100, and DAD detector UVD340U (Dionex, USA set at 254 nm) consisted of a C18 guard column (10 x 10 mm) and a Luna® 5 µm C18 analytical column (250 x 4.6 mm; Phenomenex, Germany) heated at 25 °C. The mobile phase was 5% methanol (Lach-Ner, Czech Republic) adjusted to pH 3 by o-phosphoric acid (Lachema, Czech Republic) with

a flow rate of 0.8 mL.min^{-1}. The calibration curve was determined using standard solutions of ascorbic acid p. a. (Lach-Ner, Czech Republic) at concentrations of 5, 10, 20, 60 and 100 mg.L^{-1}. The correlation coefficient of the calibration curve (R^2) was 0.9992.

The same HPLC instrumentation was used for the determination of nitrates content. The detector was set at 214 nm and KH_2PO_4 solution (c = 10 g.L^{-1}, pH 3, Lach-Ner, Czech Republic) was used as a mobile phase with a flow rate of 1 mL.min^{-1}. The calibration curve was determined using standard solutions of sodium nitrate p. a. (Sigma, Japan) at concentrations of 10, 30, 50 and 100 mg.L^{-1}. The correlation coefficient of the calibration curve (R^2) was 0.9957.

Statistical analysis

Linear regression equations, regression coefficients (R^2) as well as other results (means and standard deviations) were calculated from the data using Microsoft Office16 Excel. The differences between samples were evaluated using a one-way analysis of variance (ANOVA). Scheffe's test was used to calculate statistically significant differences between samples using statistical software Statistica 12 (StatSoft Inc.). For all statistical tests, a 5% level of significance was used.

RESULTS

Ascorbic acid content

The data from the analysis of ascorbic acid in fruit and vegetable samples are in Table 1. Concerning the fruit samples, the values of vitamin C content in plums did not vary much and was around 5 mg.100g^{-1} of the sample (from 4.24 to 6.38 mg.100g^{-1}). There was no statistical difference in vitamin C content between plums from farmers' markets and those from supermarkets. Similarly, in apple samples, the vitamin C content was less the same, ranging from 3.56 to 5.86 mg.100g^{-1} of sample and there was no difference between samples from local producers and those from supermarkets. In contrast, all strawberry samples from farmers' markets had a significantly higher vitamin C content than supermarket samples (the maximum of all analyzed samples was 70.53 mg.100g^{-1}, the minimum was 39.98 mg.100g^{-1}).

Considering vegetables, the vitamin C content in the spinach samples (ranging from 23.98 to 42.46 mg.100g^{-1}) was around an average of 30 mg.100g^{-1} and there were no differences between the market sources. There were differences in the case of tomatoes (18.00 to 32.54 mg.100g^{-1}) or red peppers samples (58.57 – 141.26 mg.100g^{-1}) from farmers' markets and supermarkets. Samples of red peppers from the supermarket had significantly higher vitamin C contents than red peppers from farmers' markets, but the opposite was true for tomatoes. In farmers' tomatoes, the average content of ascorbic acid was by 6 mg.100g^{-1} higher than in tomatoes from supermarkets. In the overall comparison of fruit and vegetables, the highest content of vitamin C was found in red pepper and the lowest in apples and plums. The major determinant of vitamin C content is the species of plant (p <0.0001) rather than where the fruit or vegetable is purchased (p = 0.9140).

Nitrates content

The levels of nitrates in samples are seen in Table 2. In the case of fruit samples, the plum samples contained from 1.80 mg.kg^{-1} to 5.00 mg.kg^{-1} of nitrates. One sample from the supermarket contained up to twice as many nitrates as the sample from the farmers' markets. The nitrates content of plum samples from supermarkets varied quite a lot from 3.25 to 5.00 mg.kg^{-1}. Similarly, apple samples from farmers' markets had significantly fewer nitrates than samples from supermarkets. The highest nitrate content was measured in a sample from the supermarket (13.02 mg.kg^{-1}), while the lowest nitrate content was measured in one sample from the farmers' market (6.36 mg.kg^{-1}). In contrast, there was no difference in nitrates content (110.17 – 131.90 mg.kg^{-1}) between strawberry samples from farmers' markets and supermarkets.

Comparing the monitored vegetable samples, there was a significant difference between spinach samples from farmers' markets and supermarket samples. The nitrate content of samples from farmers' markets was less variable (1332 – 1509 mg.kg^{-1}) than their content in samples from supermarkets (from 1084 to 2969 mg.kg^{-1}). The same was observed in the case of tomatoes (the values ranged from 37 to 53 mg.kg^{-1}) where the average measured nitrate content in samples from supermarkets was significantly higher (by 10 mg.kg^{-1}) compared to samples from local producers. On the contrary, red peppers did not show any difference between the sources where they were purchased. The nitrates contents were quite similar (around 35 mg.kg^{-1}), except for one sample from the farmer's market (71 mg.kg^{-1}).

The levels of nitrates differed significantly among all analyzed samples from supermarkets and farmers´ markets (p = 0.0349) and also among the assayed kinds of fruit and

Table 1 Ascorbic acid content in fruit and vegetables from supermarkets and farmers' markets.

Fruit or vegetable	Ascorbic acid content		
	Supermarkets (mg.100g^{-1} ±*SD*)	Farmers' markets (mg.100g^{-1} ±*SD*)	*p*-value
Apple	4.86 ±1.00	5.58 ±0.16	0.0613
Plum	5.32 ±0.95	5.25 ±0.50	0.8664
Strawberry	52.09 ±9.01	66.35 ±3.84	**0.0008**
Tomato	22.40 ±3.20	28.40 ±4.55	**0.0076**
Spinach	28.07 ±4.04	32.51 ±7.67	0.1199
Red pepper	105.53 ±26.56	78.92 ±14.99	**0.0253**

Note: *p*-values numbers marked in bold indicate numbers that are significant on the 95% confidence limit.

Table 2 Nitrates content in fruit and vegetables from supermarkets and farmers' markets.

Fruit or vegetable	Nitrates content		*p*-value
	Supermarkets (mg.kg^{-1} ±*SD*)	Farmers' markets (mg.kg^{-1} ±*SD*)	
Apple	11.84 ±1.82	7.90 ±1.40	**0.0002**
Plum	4.41 ±0.91	2.02 ±0.50	**0.0001**
Strawberry	122.39 ±7.95	116.63 ±5.90	0.1193
Tomato	42.55 ±4.35	52.33 ±3.60	**0.0002**
Spinach	2052.71 ±760.05	1391.36 ±148.87	**0.0242**
Red pepper	38.57 ±8.52	46.26 ±17.32	0.2767

Note: *p*-values numbers marked in bold indicate numbers that are significant on the 95% confidence limit.

vegetables (p <0.0001). The highest amount was in spinach, the least in plums.

DISCUSSION

Many factors are affecting the level of vitamin C in fruit and vegetables and, therefore, its content can vary a lot. From this point of view, our experimentally measured values are generally in line with literature data. Of the assayed samples, the highest content of vitamin C was in red peppers. Its average contents were 78.9 mg.100g^{-1} (from farmers' markets) and 105.5 mg.100g^{-1} (from supermarkets). This is less than observed in other studies. **McCance and Widdowson (2014)** reported an average content of 120 mg.100g^{-1} of vitamin C in red peppers in their study; **Lee and Kader (2000) and Saxholt et al. (2008)** reported values up to 151 mg.100g^{-1}. Strawberries had the second-highest average vitamin C content of 52 mg.100g^{-1} (supermarket) and 66 mg.100g^{-1} (farmers' markets). Similar results were obtained by **Lewin (1976)**, **Lee and Kader (2000),** and **Saxholt et al. (2008)**, who reported vitamin C levels in strawberries ranging from 35 to 60 mg.100g^{-1}. Vitamin C levels in strawberries are comparable to those in citrus fruits (40 – 50 mg.100g^{-1}) **(Lee and Kader, 2000)**.

Spinach is a vegetable with a high content of vitamins and minerals. Our value for vitamin C content was similar to values observed by **Bureau et al. (2015)** (23.7 mg.100g^{-1}). The vitamin C content we found in tomatoes is following literature values, 30 – 35 mg.100g^{-1} **(Lewin, 1976)**. According to **George, Kaur, Khurdiya, et al. (2004)**, however, the vitamin C content of tomato pulp may be 84 to 324 mg.100g^{-1}. The deviations of some of our results for the content of vitamin C from the data in the literature may have been affected by analyzing samples purchased in the autumn and the way of their storage **(Matějková and Petříková, 2010)**.

Our results indicated that only in the case of strawberries, tomatoes, and peppers from farmers' markets and supermarkets were significant differences in vitamin C content found. The biggest difference was in strawberry samples. Strawberries from farmers' markets contained 27.4% more vitamin C than strawberries from supermarkets. In contrast, the content of vitamin C in plums from farmers' markets and supermarkets was almost identical. There is not much information in the literature comparing products from farmers and supermarkets. Studies are usually focused on the differences between organic and conventional fruit and vegetables and their findings are often controversial **(Silva et al., 2018; Andrade et al., 2017)**. **Andrade et al. (2017)** tested the quality of strawberries grown in organic and conventional systems. The content of vitamin C in organic strawberries was (49.07 mg.100g^{-1}) and in conventional strawberries (52.32 mg.100g^{-1}). According to these authors, the content of vitamin C in strawberries does not differ significantly between the conventional and organic systems. The authors state that vitamin C content depends on many factors, including variety, ripeness, growing conditions and harvest time. These factors could then lead to significant variations in results, both between studies and within studies.

Esch et al. (2010) looked for differences in vitamin C content between organic and conventional fruits.

The tested fruits were oranges, mangoes, kiwi, lemons, gala apples and red apples. Of these six fruits, the only lemon showed a significant difference between organically (higher content) and conventionally grown samples. This study confirmed that the content of vitamin C depends on several factors, not just the way of cultivation. **Wunderlich et al. (2009)** reported that pre- and post-harvest conditions have a major effect on vitamin C levels in vegetables.

Based on our results, the hypothesis that fruit and vegetables from farmers' markets have a higher vitamin C content can, therefore, neither be accepted.

Fruit and vegetables also contain substances that could have a negative impact on human health. For this reason, the second half of this work was focused on nitrate content in fruits and vegetables from farmers' markets and supermarkets. It can be stated that there were significant differences in the nitrates content among the assayed kinds of fruits and vegetables.

According to **Prugar (2008)** and **Colla et al. (2018)**, fruit and vegetables can be divided into four categories according to nitrates content. The highest nitrate content is in leafy vegetables such as spinach. **Maynard et al. (1976)** and **Santamaria (2006)** reported that nitrates accumulate primarily in leaves, while lower nitrate concentrations are present in roots, tubers, seeds and fruits. For this reason, leafy vegetables (spinach, lettuce, parsley, etc.) are considered the most important source of nitrates. Tomatoes and peppers belong to the group with nitrates content of less than 250 mg.kg^{-1} **(Colla et al., 2018)**.

Maximum levels for nitrates content are given by **Commission Regulation (EC) No 1881/2006** only for salad and spinach. The maximum limit for spinach is set in two categories, i.e. 3500 mg NO_3.kg^{-1} for the harvest from 1 October to 31 March and 2500 mg NO_3.kg^{-1} for the harvest from 1 April to 30 September. The highest measured nitrates content in the spinach sample in our case was

2969 mg.kg^{-1}. As the purchase of the samples took place in October, it can be assumed that the maximum limit for nitrate content was not exceeded.

Prugar (2008) and **Colla et al. (2018)** classified spinach into the "very high nitrates category", i.e. more than 2000 mg.kg^{-1}. In this study, the results of spinach from farmers' markets with average nitrates content of 1391 mg.kg^{-1}, would fit to the "high nitrates category" (i.e. 1000 – 2000 mg.kg^{-1}). Supermarket spinach contained 47.5% more nitrates than spinach from farmers' markets. This difference was most likely due to different growing conditions. Fruit and vegetables from wholesalers come very often from greenhouses.

The main factors contributing to the increased nitrate concentration are temperature, lack of light and strong fertilizer concentration. In contrast, products from farmers' markets are usually grown in the open air in fields with enough light. Moreover, it is best to buy vegetables from the afternoon harvest. In the morning, the nitrate content in fruits and vegetables is higher than in the evening. It is therefore recommended to harvest agricultural products in the afternoon **(Sorensen, Johansen and Poulsen, 1994; Colla et al., 2018)**.

Muramoto (1999) conducted a similar study with iceberg lettuce, Roman lettuce and spinach from supermarkets (conventional cultivation) and farmers' markets (organic cultivation). This author concluded that samples of spinach from the supermarket (average nitrates content 2540 mg.kg^{-1}) had a significantly higher nitrates concentration than samples from farmers' markets (average nitrates content 1810 mg.kg^{-1}). **Muramoto (1999)** also suggested that the use of nitrogen fertilizers may be the main cause of this difference. In other studies, **Barker (1975)** and **Stopes et al. (1989)** reported a positive correlation between the number of nitrogen fertilizers and nitrates accumulation in spinach.

Compared to vegetables, the fruit samples had much lower nitrates content. **Bahadoran et al. (2016)** reported an average of 27 mg nitrates per 100 g plums in their study. The highest nitrates content of our fruit samples was found in strawberries, which belong (together with bananas) to a "higher nitrates category". **Walker (1990)** reported up to 150 mg of nitrates in 1 kg of strawberries in his study. We observed average nitrates content of strawberries from 116.63 mg.kg^{-1} (from farmers' markets) to 122.39 mg.kg^{-1} (from supermarkets). The explanation for the high nitrate content could be the presence of a thin, porous layer on the surface of strawberries, their frequent fertilization, or the way of growing in greenhouses where the lack of light causes the accumulation of nitrates.

In the case of our samples, no significant difference was found between strawberries from supermarkets and farmers' markets. This observation is consistent with the study of **Bordeleau et al. (2002)**, who also examined the difference in nitrates content of strawberries from farmers' markets and supermarkets. Other samples – plums, red pepper, tomatoes, and apples contained less than 250 mg of nitrates per kg, thus belonging to the low nitrate fruit and vegetable category according to the nitrates content classification **(Prugar, 2008; Colla et al., 2018)**.

In three cases out of six, the fruit and vegetables tested by us from farmers' markets contained statistically lower nitrates concentrations than those purchased in supermarkets. In these cases, the hypothesis that fruits and vegetables from farmers' markets have lower nitrates content than fruits and vegetables from the supermarket can, therefore, be accepted. On the other hand, there was no significant difference between the nitrates contents of strawberries and red peppers from farmers' markets or supermarkets, whereas tomatoes had significantly higher nitrates content when bought from farmers' markets.

Studies that have found significantly lower levels of nitrates in products from farmers' markets generally explain this by a lower fertilization rate. **Bordeleau et al. (2002)** stated that mineral fertilizers have a greater effect on nitrates levels than livestock fertilizers or humus. Other important factors influencing nitrates content are weather and light conditions.

CONCLUSION

The results of this work showed that the content of ascorbic acid and nitrates differs significantly depending on the type of fruit and vegetables. The most important source of vitamin C from the assayed fruits and vegetables was red pepper. One hundred grams of it would sufficiently cover the recommended daily dose of 90 mg. Comparing the different kinds of fruit and vegetables, significant differences in vitamin C content between samples from farmers' markets and samples from supermarkets were found in strawberries, red peppers and tomatoes.

Analyses of nitrates content showed significantly lower nitrates content in spinach, apples and plums from farmers' markets than from supermarkets. The most important source of nitrates was spinach, but the content did not exceed the limit set by legislation. Compared to vegetables, the fruit samples had much lower nitrates contents. Strawberries had the highest nitrates content of the fruit samples examined.

Overall, there were significant differences in nitrate content in fruit and vegetables from supermarkets and farmer´s markets. On the other hand, differences in vitamin C content were not found.

Since the majority of studies compare the quality of organic and conventional products rather than comparing the nitrates and vitamin C contents of fruit and vegetables from farmers' and supermarkets, it would be better to focus on this area in the future. Our findings suggest that farm products may not always be a better choice in terms of vitamin C and nitrates content than fruit and vegetables purchased in supermarkets.

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INFLUENCE OF DIFFERENT STORAGE CONDITIONS ON THE OCCURRENCE OF ENTEROCOCCI IN SMEAR RIPENED CHEESES

Olga Cwiková, Gabriela Franke

ABSTRACT

The number of enterococci was monitored in smear-ripened cheeses stored under different temperature regimes. Sampling and subsequent analyses were performed on the day of manufacture (A/0 = B/0 = C/0), at the end of BBD (A/35, B/35, C/35), two weeks after BBD (A/49, B/49), and eight weeks after BBD (C/91). No statistical difference (p >0.05) was found in the numbers of enterococci in cheeses stored under different temperature regimes until the Best-Before date or at the end of monitoring after 49 and 91 days respectively. At the beginning of storage (A/0, B/0, C/0), the numbers of enterococci in cheeses were 2.3 log CFU.g^{-1}. The highest number of enterococci was recorded after 49 days of storage at 6 °C at 5.4 log CFU.g^{-1}. During storage, there was an increase (p <0.05) in the numbers of enterococci in all types of temperature regimes. Enterococci content was influenced (p <0.05) by both the storage period and storage method (temperature regime).

Keywords: *Enterococcus* spp.; smear-ripened cheese; storage period and temperature

INTRODUCTION

Enterococcus sp. is a gram-positive bacterium found in various places: it is a commensal living in the digestive tract of animals, insects and humans **(Li et al., 2017)**, often found in fermented foods such as fermented dairy and meat products, in soil, water and plants **(Lebreton et al., 2014; Fuka et al., 2017)**. Enterococci are found in large quantities in traditional products made from raw milk, particularly in cheeses. Enterococci are associated with traditional European cheeses manufactured in Mediterranean countries, such as Greece, Italy, Spain and Portugal, from raw or pasteurized goats', ewes', water-buffalos', or bovine milk **(Moreno et al., 2006)**. Although their presence is generally considered to be due to inadequate hygiene conditions during processing **(Gelsomino et al., 2002)**, they can act as natural starter cultures in the production of various types of cheeses **(Giraffa, 2003)**. These are artisan cheeses produced in Southern Europe, e.g. Venaco cheese **(Casalta and Zennaro, 1997)**. Enterococci are found in cheeses made from both raw and pasteurized milk because they survive the pasteurization temperature **(İspirli et al., 2017)**. The resistance of enterococci to pasteurization temperatures and their ability to adapt to different substrates and different growth conditions not only leads to their discovery in foods made from raw materials (milk, meat) but also in foods obtained by the thermal process. They are therefore able to survive the conditions of food production. They can also contaminate finished products **(Hanchi et al., 2018)**. However, their incidence in traditional cheeses made from raw cow's milk of high microbiological quality is low **(Garabal et al., 2008)**, which is likely to result in the loss of some taste attributes.

In terms of pathogenicity and antibiotic resistance, as well as biogenic amine production, enterococci in food, of course, are not desirable microorganisms.

However, some enterococci are used in the industry **(Ogier and Serror, 2008)** because of their biochemical properties suitable for technological applications **(Centeno and Carballo, 2015)**. Some strains have been designed as complementary starting microbiota **(Franz et al., 2001; Moreno et al., 2003)**, *E. durans, E. faecalis* and *E. faecium* are also marketed as probiotic cultures **(Centeno and Carballo, 2015)**, show antimicrobial effects against alimentary pathogens, which is related to the presence of genes encoding enterotoxin production **(İspirli et al., 2017)**. For this reason, *Enterococcus* sp. is one of the most controversial genera belonging to lactic acid bacteria **(Lebreton et al., 2014)**. Enterococci can adapt to adverse environmental conditions. They are also known for their antibiotic resistance. The most important enterococcal diseases include urinary tract infections, nosocomial infections and superinfections, meningitis, bacterial endocarditis and bacteraemia **(Gardini et al., 2001; Arias and Murray, 2008)**.

Scientific hypotheses

Hypothesis 1: Enterococci survive the freezing process and their number does not change significantly during the freezing of cheeses.

Hypothesis 2: The duration of the storage of cheeses affects their safety in terms of enterococcal content.

MATERIAL AND METHODOLOGY

The smear ripening cheese for testing was delivered as small rounds (diameter 45 mm, height 10 mm), where a 100 g package contained 5 pieces of these round portions. Before shipment, the cheeses ripened for 7 days, while the best before date (BBD) on the package was 28 days. A total of 5 batches of the product were analyzed. Each batch of the samples was made from different pasteurized milk. Analyzed smear-ripened cheese is produced from fat-free sour curd. To the curd was added water, neutralization salts and *Candida valida* and bacteria *B. linens*. After the formation of cheese by a drying process, which allows for the reproduction of oxidizing yeasts *Torulopsis* spp. and *Candida* spp., which creates a suitable environment for the onset of action of proteolytic bacteria *B. linens* that during maturation forms on the surface of cheeses which are golden yellow to orange sebum in color.

The samples were supplied by the cheese manufacturer and were divided into three groups designated as (A), (B) and (C) then stored in different temperature regimes. Sampling and subsequent analyses were performed on the day of manufacture (A/0 = B/0 = C/0), at the end of BBD (A/35, B/35, C/35), two weeks after BBD (A/49, B/49), and eight weeks after BBD (C/91).

Samples in the temperature regime (A) were stored at 6 °C after production for the entire BBD (7 days before and 28 days after shipment), i.e. 35 days. These cheeses were then stored for 14 days after the BBD expired at 6 °C, i.e. 49 days in total (6 °C/35 days → 6 °C /14 days). Samples in the temperature regime (B) were stored at 6 °C after production for 28 days (7 days before and 21 days after shipment), i.e. 28 days, then they were frozen and stored at -18 °C for 7 days. Subsequently, after BBD (35 days), the cheeses were stored at 6 °C for 14 days, i.e. 49 days in total (6 °C/28 days → -18 °C/7 days → 6 °C/ 14 days). Samples in the temperature regime (C) were stored at 6 °C after production for the entire BBD (7 days before and 28 days after shipment), i.e. 35 days. They were then frozen and stored for 49 days (7 weeks) at -18 °C. After 49 days of freezing, the cheeses were stored at 6 °C for 7 days, i.e. 91 days in total (6 °C/35 days → -18 °C/ 49 days → 6 °C/7 days).

The analyses were carried out in the laboratory of the Institute of Food Technology, Faculty of AgriSciences of Mendel University in Brno according to **STN EN ISO 7218:2007 (2008)**. For one analysis, 3 consumer packages were always used. 10 g of each 100 g package of cheese was taken with a sterile scalpel by cutting a section through the sample through all the wheels; the sample contained the center and the edge of the cheese **(Figure1)**.

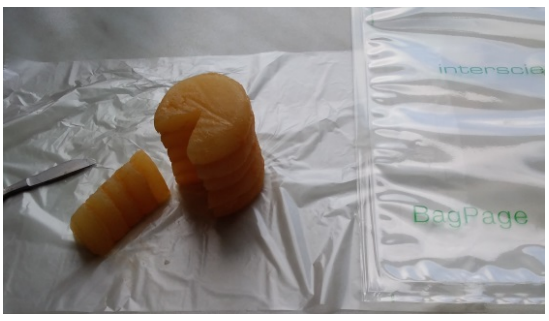

Figure 1 Sample preparation (smear ripened cheese).

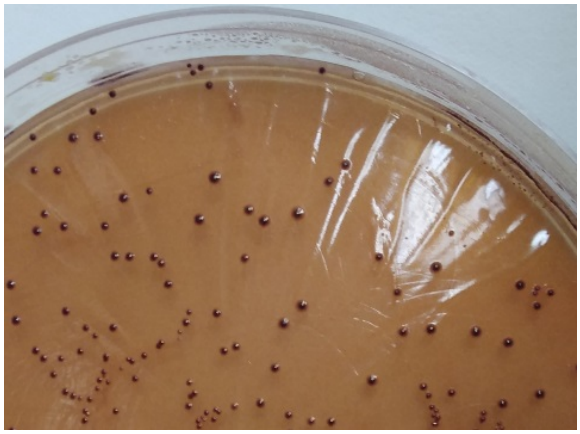

Figure 2 *Enterococcus* spp. in Slanetz-Bartley agar (isolated from smear ripened cheese).

Enterococci were determined on the Slanetz-Bartley agar (soil composition according to **STN EN ISO 7899-2 (2001)**, aerobically grown at 37 °C for 48 h **(Figure 2)**. Confirmation was made by growth on bile esculin agar (composition according to **STN EN ISO 7899-2 (2001)**; incubation at 44 °C for 24 h) and the catalase test (negative).

Statistical evaluation was performed in the Statistica Statsoft program (version 12) and Microsoft Excel 2010. Basic statistical characteristics, such as mean and standard deviation of the mean were calculated.

To compare the *Enterococcus* spp. content during the storage period within the given temperature regime, a simple scattering analysis method (ANOVA) including the Duncan post-hoc test was used. Normality was tested by the Shapiro-Wilk test.

The proportion of factors (temperature and storage period including interactions) on the total variability of BA content in cheese was calculated using the general linear model (two-way ANOVA with interactions).

RESULTS AND DISCUSSION

Influence of storage temperature on the number of enterococci

No statistical difference (p >0.05) was found in the number of enterococci in cheeses stored under different temperature regimes until the Best-Before date or at the end of monitoring after 49 and 91 days **(Figure 3)** respectively. Enterococcal counts were 4.7 log CFU.g^{-1} when stored under the regime (A) until BB date after 35 days of ripening, 4.8 log CFU.g^{-1} when stored under temperature regime (B) and 4.7 log CFU.g^{-1} under the regime (C). At the end of monitoring, 5.4 log CFU.g^{-1} of enterococci was detected in cheeses after storage for 49 days under the temperature regime (A), 5.2 log CFU.g^{-1} when stored under the regime (B) and 5.0 log CFU.g^{-1} under the regime (C) after 91 days.

Enterococci are highly resistant to environmental conditions, grow over a wide temperature range (25 to 45 °C), tolerate pH from 4.8 to 11.0, the salt content of

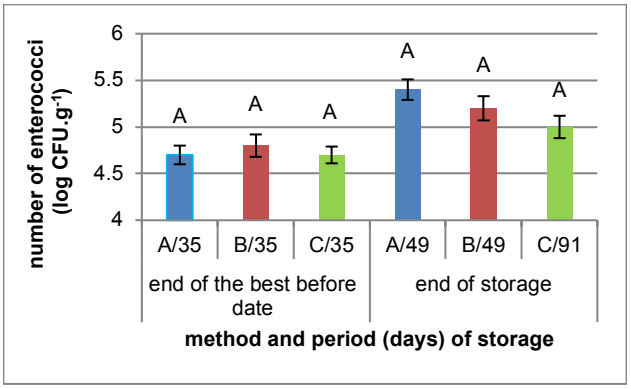

Figure 3 Comparison of the the number of enterococci (log CFU.g^{-1}) in smear ripened cheeses stored in different temperature regimes (A, B, C) and analysed at the end of the best before date after 35 days of ripening and at the end of storage after 49 or 91 days of ripening. Note: A/35: storage at 6 °C/35 days, B/35: Storage at 6 °C/28 days and at -18 °C/7 days, C/35: storage at 6 °C/35 days) (A: storage at 6 °C/35 days and at 6 °C/14 days, B: storage at 6 °C/28 days and at -18 °C/7 days and at 6 °C/14 days, C: storage at 6 °C/35 days and at -18 °C/49 days and at 6 °C/7 days; n = 15.

6.5 percent, bile presence of 40 percent and survive at 60 °C for 30 minutes **(Domig, Mayer and Kneifel, 2003)**. **López-Díaz et al. (2000)** indicated a wider range of growth temperatures from as low as 10 °C.

Due to their high resistance, they survive and tolerate temperature changes well, as shown in **Figure 3**. **Bockelmann et al. (2005)** reported that the usual number of enterococci on the surface of smear-ripened cheeses is <6 log CFU. cm^{-2}. Higher counts of enterococci compared to our study, >8 log CFU. cm^{-2}, were also detected by **Amato et al. (2012)** for fully ripe smear-ripened cheeses when stored at 4 to 8 °C. **Komprda et al. (2012)** also recorded higher counts compared to our study (depending on the season) until the Best Before date (42 days after production) when stored at 5 °C, namely 6 to 7 log CFU.g^{-1}, and 5.2 to 6.4 log CFU.g^{-1} after 66 days after production, wherein the lower value corresponds to the counts of enterococci that we found in our experiment at the end of the monitoring under the regimes (A/49), (B/49) and (C/91). Also, **Fontana et al. (2010)** reported higher counts of enterococci in smear-ripened cheese compared to our study, namely 7.2 log CFU.cm^{-2}. According to **Schneller, Good and Jenny (1997)**, the distribution of enterococci in cheese may be inhomogeneous.

Freezing is not the cause of the devitalization of all cells **(Görner and Valík, 2004)**. As reported by **Kadlec, Melzoch and Voldřich (2012)**, during freezing enzymatic reactions proceed slowly. Because they are mesophilic bacteria, according to **Šilhánková (2008)**, when the temperature falls below the optimal growth level, the rate of propagation drops sharply and eventually stops, although the sensitivity to cold shock varies among different bacteria. The devitalizing effects of low temperatures are, according to **Drdák et al. (1996)**, more efficient in repeated freezing and thawing, but this is not the case with bacterial spores that may survive under these conditions.

Possible sources of contamination of cheeses with enterococci are the raw material (milk), production workers, production equipment, water taps, saline solutions, tanks; however, their origin in cheeses is sometimes unclear **(İspirli et al., 2017)**. **Greifová et al. (2003)** reported that the decisive enterococci contamination of milk comes from the milking equipment and plant feed. In raw milk, enterococci are clear indicators of inadequate decontamination of equipment and machinery. As thermoresistant bacteria, some species of enterococci survive required pasteurization temperature, therefore they are a normal part of pasteurized milk.

Influence of the storage period on the number of enterococci

At the beginning of storage (A/0, B/0, C/0), the numbers of enterococci in cheeses were 2.3 log CFU.g^{-1}. At the end of storage, 5.4 log CFU.g^{-1} of enterococci was detected in cheeses under (A) temperature regime, 5.2 log CFU.g^{-1} when stored under (B) regime and 5.0 log CFU.g^{-1} when stored under (C) regime conditions. During storage, there was an increase (p <0.05) in the number of enterococci in all types of temperature regimes **(Figure 4)**.

Increasing enterococcal numbers during ripening was described by **Novella-Rodríguez et al. (2004)** for goat cheeses made from raw milk, **Roig-Sagués et al. (2002)** for Spanish cheeses, **Maher and Murphy (2000)**, **Macedo et al. (2004)** and **Martuscelli et al. (2005)** for smear-ripened cheeses. Counts of enterococci increased during 60 days of ripening of cheeses made from pasteurized milk in an experiment by **Martuscelli et al. (2005)** from 3.3 ±0.2 to 8.8 ±0.3 log CFU.g^{-1}, which are much higher values compared to our study. According to **Komprda et al. (2012)**, at 5 °C enterococci counts did not change significantly in smear-ripened cheeses (p >0.05); at

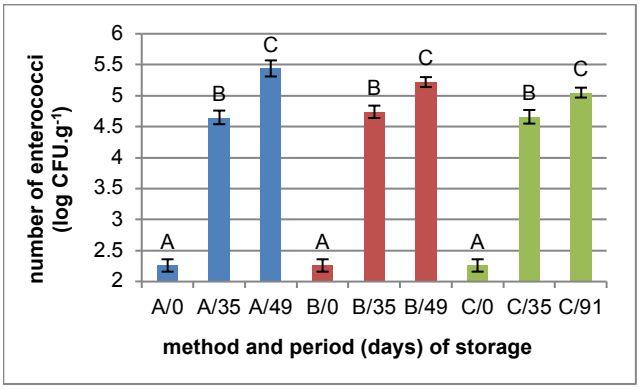

Figure 4 Comparison of the the number of enterococci (log CFU.g^{-1}) in smear ripened cheeses stored in different temperature regimes (A, B, C) and analysed on production day (0), at the end of the best before date after 35 days of ripening. Note: A/35: storage at 6 °C/35 days, B/35: storage at 6 °C/28 days and at -18 °C /7 days, C/35: storage at 6 °C/35 days and at the end of storage after 49 or 91 days of ripening: A: storage at 6 °C/35 days and 6 °C/14 days, B: storage at 6 °C/28 days and at -18 °C/ 7 days and 6 °C/14 days, C: storage at 6 °C/35 days and at -18 °C/49 days and at 6 C/7 days. Averages marked with different letters are statistically different within a given factor (storage period) (p <0.05); n = 15.

the end of ripening, the counts of enterococci were 6.1 log CFU.g^{-1}, which is more than 1 log higher than our study recorded. Although the number of enterococci in cheeses may be 1 log CFU.g^{-1}, values reaching up to 7 log CFU.g^{-1} have been found **(Pircher et al., 2007)**. Based on the experiment, **Rea et al. (2004)** concluded that the increase in enterococcal numbers in the first 6 hours (from 5 log to 7 log CFU.g^{-1}) is due to the water loss during cheese processing, which appears to be the cause of the apparent increase in enterococcal numbers. The survival of enterococci in cheese during ripening is due to a wide range of growth temperatures, tolerance to high temperatures, salt and acid concentrations **(Šilhánková, 2008)**. In contrast to enterobacteria, enterococci show a higher resistance to antagonist bacteria, which are represented by starter microorganisms **(Macedo et al., 2004)**. An increase in the number of enterococci during the ripening of smear-ripened cheeses was also described by **Calasso et al. (2016)**; after 30 days of ripening their number increased to 4.5 ±0.2 log CFU.g^{-1}, which is comparable to our experiment. Also **Schneller, Good and Jenny (1997)** noted an increase in the number of enterococci during cheese ripening. Levels of enterococci in different cheese curds range from 10^4 to 10^6 CFU g^{-1} and in the fully ripened cheeses from 10^5 to 10^7 CFU g^{-1} **(Moreno et al., 2006)**.

Enterococci are widespread in nature and get into raw milk and dairy products during production, especially from the equipment and also from the water where basic hygiene conditions are not observed **(Ogier and Serror, 2008; Li et al., 2017)**. Enterococci are commonly found in raw milk with different microbiota reported in different countries, reflecting local practices and levels of hygiene **(Čanigová et al., 2016)**. We are, however, not able to fully explain the origin of enterococci in cheeses, as the cheeses were made from pasteurized milk. Thus, enterococci originating from the gastrointestinal tract of a dairy cow should be devitalized by heat treatment. The same conclusions were reached by **Gelsomino et al. (2002)**, who found that dairy cow manure is not a source of enterococci, even though the same enterococcal strains were found in milk, cheese and dairy equipment as in dairy cow manure. On the contrary, **Görner and Valík (2004)** reported that Kielwein did not find a link between the species composition of enterococci in dairy cow manure and the milking equipment in 1997 and concluded that enterococci cannot be considered as indicators of faecal contamination but as indicators of inadequate sanitation.

This study also evaluated the contribution of the method and time of storage to the variability in the number of enterococci. Analyses carried out (two-way ANOVA with interactions) show that both the storage period and the storage method (temperature regime; **Table 1**) influenced the enterococcal content.

Table 1 Influence of individual factors (storage method, storage period) on the enterococci content (initial measured values in log CFU.g^{-1}) in cheeses.

Indicator	% explained variability			
	Storage method	Storage period	Interaction	Error
Enterococci	7*	82*	7*	4

Note: * p <0.05.

According to **Giraffa (2003)**, it is difficult to answer the question of whether the presence of enterococci in food is safe. It is necessary to carry out a more detailed identification of the individual strains present in cheeses and to evaluate the pathogenicity of these strains **(Franz, Holzapfel and Stiles, 1999)**. Due to the variety of *Enterococcus* species and their importance in food, the detection and enumeration of enterococci have become an important concern in current research activities **(Hamad, Selim and Yassin, 2019)**.

CONCLUSION

The hypothesis that enterococci survive the freezing process and their numbers do not change significantly can be confirmed. No statistical difference (p >0.05) was found in the numbers of enterococci in cheeses stored under different temperature regimes until the Best-Before date or at the end of monitoring after 49 and 91 days, respectively.

The hypothesis that the duration of the storage of cheeses affects them in terms of enterococcal content can be confirmed. During storage, their numbers increased (p <0.05). However, it should be noted that the view of enterococci is not unambiguous. On the one hand, they are used as cheese-making cultures in some countries, and on the other hand, they can cause health problems in immunocompromised individuals.

From the viewpoint of pathogenicity and resistance to antibiotics, as well as the production of biogenic amines, enterococci in foods are not desirable microorganisms.

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DETERMINATION OF SELECTED TERPENIC SUBSTANCES IN GRAPES AND WINE OF THE CULTIVAR PÁLAVA

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ABSTRACT

The presented study is focused on the determination of the content of terpenic substances in grapes and subsequently produced a wine of Czech variety Pálava (the Czech Republic, wine region Morava), which is a protected landscape with long-term tradition and culture. The aim of this study was the analysis of the aromatic profile of the cultivar Pálava, which was fermented by the original yeast strains from the Moravian wine region. Larger amounts of flavoring substances occur in grapes as bound flavoring substances, most often in the form of glycosides. One of the basic groups of aromatic substances is monoterpenes. The content of twelve free and bound terpenic substances was measured by the GC-MS method, namely linalool, ho-trineol, α-terpeniol, β-citronellol, nerol, geraniol, furan linalool oxide 1, furan linalool oxide 2, nerol oxide, epoxylinalol 1, epoxylinalol 2 and 2,6-dimethyl-3,7-octadiene-2,6-diol. The results were statistically compared by using a simple descriptive statistical method and ANOVA method. We noted a difference between the content of free and bound terpenes was very significant ($p \leq 0.05$). We found that using uncommercial yeasts could have an effect on the content of the volatile and terpenic compounds in wines. An important finding was that in fresh berry extracts there was a higher proportion of bound terpenes than free terpenes. The results have shown that the production technology of wine and the fermentation process has a clear impact on the content of the substances.

Keywords: terpens; grape; wine; GC-MS

INTRODUCTION

Pálava is a Moravian wine grape cultivar, named after the Protected Landscape Area of Pálava, which was created by crossing the cultivars Traminer Red and Müller Thurgau. The cultivar was bred by Ing. Josef Veverka, who started this breeding in 1953 at the Breeding Station in Velké Pavlovice and later continued in Perná. The cultivar has been entered in the State Variety Book in 1977 **(Kuneš et al., 2015)**.

The Pálava Protected Landscape Area (PLA) means a rich natural and cultural heritage. The vineyards cover approximately 16% of the total size of PLA **(Miklín and Smolková, 2011)**.

Despite the busy tourism in the area, wine and viticulture are an important phenomenon and the most important wine-growing area in the Czech Republic **(Miklín, 2012)**. Shrubs are moderately lush with dense leaves. The leaves are medium-sized, usually shallow-lobed. There is dense hair on the back, the blade surface is indefinitely wavy and leathery. The flowers are two-sexed, self-dusting, and five-flowered. The fringes are medium-sized, 100 – 160 mm long, conical, and sometimes winged **(Hakl et al., 2007)**.

The berries are light red with a gray tinge and have an oval shape with an average length of 14 mm. The skin is firm and the flesh is juicy, aromatic, with a spicy flavor. The average weight of the bunch is 169 g, the average weight of the berries is 1.1 g. It is moderately resistant to winter frosts and less resistant to spring frosts due to early budding. It is prone to attack by insects because it has very thick shrubs due to pecks. Resistance to other fungal diseases is average **(Kraus et al., 2005)**.

By **Butnariu and Butu (2019)** wine is one of the best selling agricultural products and by **Kong et al. (2019)** grapes (*Vitis vinifera* L.) are economically important fruit crops worldwide. The most important factors affecting the aroma in Muscat and non-Muscat cultivars are terpenic and aliphatic alcohols **(Matujašević et al., 2019)**.

Among the attributes that affect the marketability of wine, there are the production method and technological processes, such as the fermentation of fresh grapes, the application of the quantity and type of yeast, etc.

The study is focused on the analysis of the aromatic profile of the cultivar Pálava, which was fermented by the original yeast strains from the Moravian wine region. Using gas

chromatography with mass detection (GC-MS) 12 terpenic substances were determined, which are important for shaping the character of the wine.

Scientific hypothesis

The difference between the results of the determination of free and bound terpenes in berries will be significant.

The content of the individual terpenes will be higher for the bound terpenes than for the free terpenes.

The content of terpenic substances is higher in fresh berries than in samples of the wine.

MATERIAL AND METHODOLOGY

The measured contents in grapes and wine were determined by extraction with methyl t-butyl ether. The weight of one sample was 100 grams of the cultivar Pálava. Samples of berries and wines were taken from the wine region of Moravia. Wine sampling was carried out one month before the end of the fermentation. At the same, we used a control sample which contained the only methyl t-butyl ether.

To fulfill the objectives of this study, there were used technology Shimadzu GC-17A, Autosampler: AOC – 5000, Detector: QP-5050A.

The results have been processed by using the software GC solution. Program LabSolutions, GC MS. Version 1.20. The separation conditions have been set as follow: Column: DB-WAX 30 m x 0.25 mm; 0.25 µm stationary phase (polyethylene glycol).

An injection volume of sample: 1 µL split ratio 1:5.

Carrier gas flow rate He: 1 mL.min^{-1} (linear gas velocity 36 cm.sec^{-1}).

The injection chamber temperature was set at 200 °C.

The initial temperature of the 45 °C column was maintained for 3.5 minutes, followed by a temperature gradient: up to 90 °C by 15 °C.min^{-1}, up to 135 °C by 6 °C.min^{-1}, up to 207 °C by 9 °C.min^{-1}, up to 252 °C by 15 °C.min^{-1}.

The final temperature was held for 5 min. The total analysis time was 30 minutes. The detector operated in SCAN mode with an interval of 0.25 s in the range of 14 – 264. Detector voltage at 1.5 kV.

Substances were identified based on mass spectrum and retention time. Quantification was performed by comparing the sample peak area and the external standard with the correction for the internal standard.

The used analytical method, gas chromatography-mass spectrometry (GC/MS) was similar to the scientific study by **Pedersen et al. (2003)** and **Dziadas and Jeleń (2010)**.

Basic measured parameters of analyzed grapes were: weight 50 berries (76 g); red. sugar (22.8 °NM); pH (3.27); titric acids (7.6 g.L^{-1}).

The basic measured parameters of the analyzed wine were: red. sugar (0.1 g.L^{-1}), content of alcohol (11.7%); pH (3.5), titr. acids (7.94 g.L^{-1}).

Statistical analysis

After measuring terpene content in the samples, the results were statistically evaluated using simple descriptive statistics and the ANOVA method- one-factor method (without repetition). For statistical analysis, we used statistical software MS Excel (v.16.0, Microsoft-Windows).

RESULTS AND DISCUSSION

In the study, **González-Rodríguez et al. (2011)** three new commercial fungicides have been applied to wines according to GAP, which due to the aromatic structure of white wines. In the presence of fungicidal residues, the scent manifestation of geraniol was significantly reduced, thereby significantly reducing the floral nuance. However, it has not been shown that these fungicides have affected the formation of 1-hexanol and cis-3-hexen-1-ol formed during the fermentation processes, including harvesting, transport, crushing, and pressing.

Aim of the study **Zeleňáková et al. (2011)** was to examine of factors (manufacturer, temperature and storage time) influencing the variability of yeast amount and pH changes in bottled white wines. They discovered that storage time did not significantly impact the amount of yeast in wine (p = 0.5507).

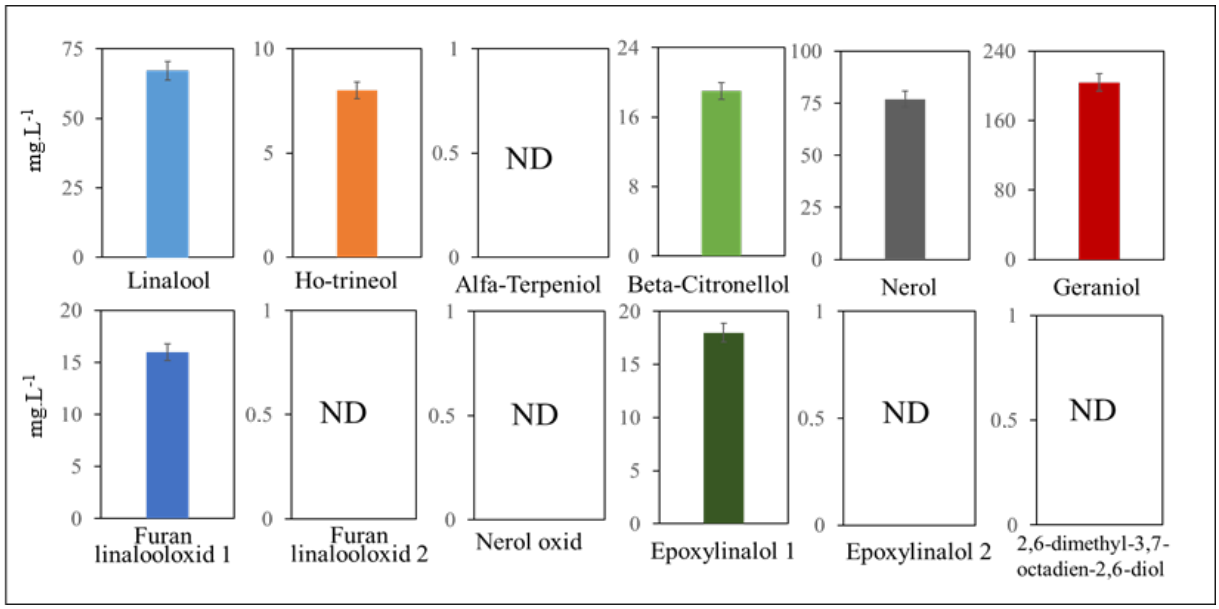

Figure 1 Values of the content of free terpenic substances in berries of the cultivar Pálava.

According to **Synos, Reynolds, and Bowen (2015)**, the use of various yeasts affects the content of ethyl isobutyrate, isoamyl acetate, 3-octanone, hexanoic acid, nerol, phenylethanol, and vitispiran. This claim was based in particular on the formation of various volatile substances found in wine. Using commercial yeast, the type of wine, berry processing, and fermentation is a factor for increasing the incidence of terpenes. **Lengyel and Panaitescu (2017)** studied the accumulation of free volatile terpene flavors (FVT) and bound precursor terpene flavors (BPT) under the action of 12 selected wine yeast strains. The 12 strains used in the alcoholic fermentation resulted in the determination of considerable amounts of terpene flavor compounds, between 370 $\mu g.L^{-1}$ and 1100 $\mu g.L^{-1}$, which recommends their use in making quality wines. By **Kačániová et al. (2019)** the wine grape berries share a complex microbial ecology including filamentous fungi, yeasts and bacteria. The study **Holešinský et al. (2020)** focused on the isolation of a consortium of microorganisms from spontaneously fermenting that naturally contain lactic acid bacteria, non-saccharomyces yeasts, and saccharomyces yeasts. The smallest amount of ethanol was formed from the isolates containing *Hanseniaspora uvarum*, while *Candida sake* isolate produced the lowest amount of hydrogen sulfide and *Zygosaccharomyces bailii* produced the highest. The killer proteins of *Pichia* spp. on the wine spoilage yeasts, studied by **Błaszczyk, Satora and Sroka (2015)**, are known for their broad spectrum of antifungal activity including pathogens such as *Candida albicans*. In this study, there was stated that the killer toxins could be used in the food industry as selective tools to control infections during the fermentation of wine. We agreed with this opinion. There are a lot of studies that determined the presence of microorganisms in wine and grape berries, namely e. g. **Drożdż et al. (2015)**; **Snopek et al. (2019)**; **Felšöciová, Kačániová and Vrábel (2020)**; **Kunová et al. (2020)**.

Yang et al. (2019) demonstrated that seventeen terpene glycosides were quantified in grapes and wines as pentosyl-glucopyranoside, the content of which ranged from 804 to 836 $\mu g.kg^{-1}$, and from 155 to 192 $\mu g.L^{-1}$. Eight free terpenes were present in wines with their content ranging from 40.1 to 59.7 $\mu g.L^{-1}$.

Compared to the above studies, it was noted that linalool (it has a floral aroma with a touch of lemon and spices) content was significantly higher for bound terpenic substances compared to free terpenic substances in berries ($p \leq 0.05$). Similar results were also observed in the determination of ho-trienol, α-terpeniol, geraniol, furan linalooloxid 1 and 2, nerol oxide, epoxylinalol 1, and 2, and ultimately 2,6-dimethyl-3,7-octadien-2,6-diol were not detected (Figure 1 and 2). To compare and obtain more accurate data, we measured each sample in three repetitions.

A statistically significant relationship between the individual measured attributes in the samples was demonstrated ($p \leq 0.05$).

Kostrz and Satora (2018) stated that the most common terpenes in wines are nerol (associated with citrus, magnolia aroma), citronellol (rose aroma), geraniol (with pleasant rose, geranium aroma), limonene (orange, citrus aroma), linalool (associated with floral, herbal, lavender), citral (with the smell of citrus) and β-ionone (with pleasant seaweed, violet, flower, raspberry).

By **Somkuwar et al. (2019)** among the studied varieties, Nielluccio wine recorded the highest concentration of total volatile compounds (191.53 $mg.L^{-1}$) while, it was least in Caladoc wines (15.45 $mg.L^{-1}$).

Comparing the measured terpene contents with the study **Matujašević et al. (2019)** the total relative content of terpenes in the control sample from 4.80% (Banat Muscat) to 24.78% (Radmilovac Muscat), in the sample with a lower dose of the enzyme (0.3 $g.kg^{-1}$) from 8.05% (Italia Muscat) up to 50.85% (Early Muscat) and in the sample with the higher dose of the enzyme (0.7 $g.kg^{-1}$) from 11.07% (Italia Muscat) to 34.78% (Radmilovac Muscat). The content of terpenic substances increased by an application of enzymes.

From this aspect, it can be deduced that in this way an increase in volatile and terpenic substances can be

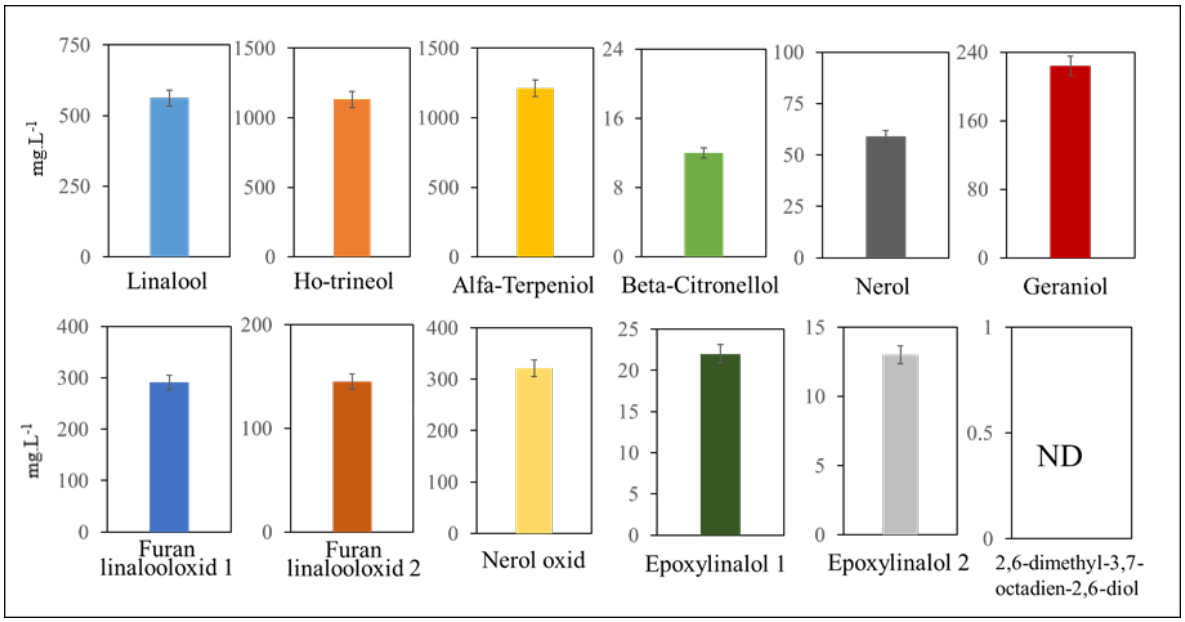

Figure 2 Values of content of bound terpenic substances in berries of cultivar Pálava.

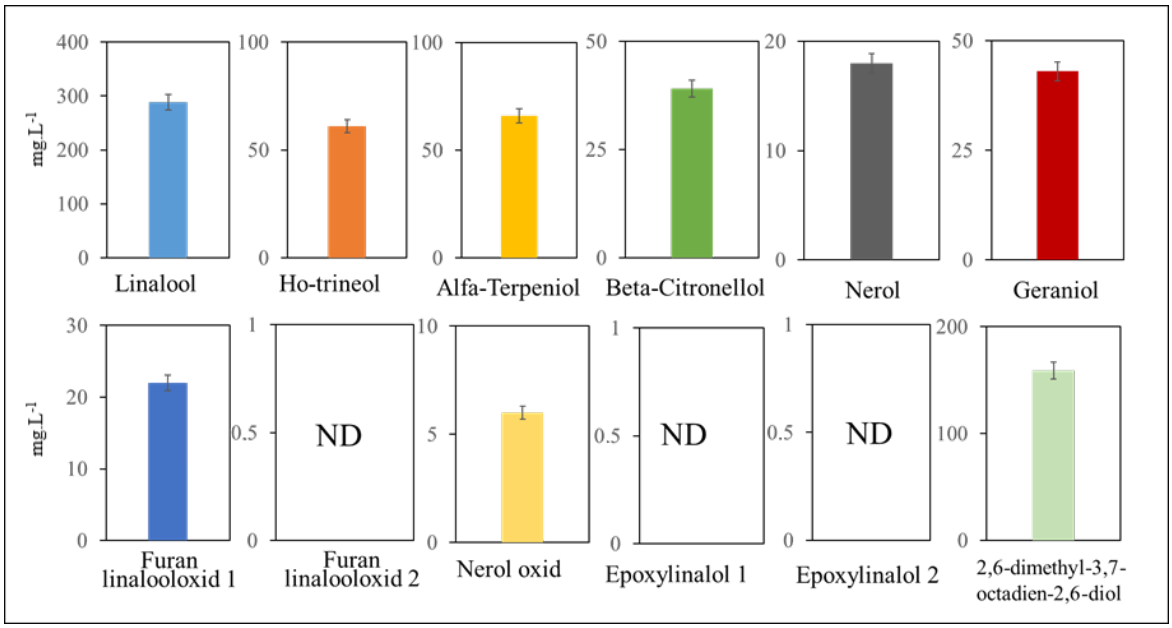

Figure 3 Values of total terpenic substances in wine of the cultivar ´Pálava´.

achieved.

By comparing results which are shown in Figure 3, we noted that despite β-citronellol, the content of terpenic substances was very low. 2-6-dimethyl-3,7-octadien-2,6-diol were measured only in wine samples wherein the samples of fresh berries were not detected.

We demonstrated a statistically significant meaning between the measured values of terpene compounds in the wine ($p \leq 0.05$).

Baron et al. (2017) deal with the determination of the content of both free and bound terpenes in berries and wine. They declared that the terpene content in wine increased gradually with the period of maceration. The highest and the lowest amounts of terpenes were recorded after 24 hours of maceration and no maceration, respectively. We agreed with this opinion because the length of maceration affects the content of terpens in wine. Otherwise, in the study **Blagoeva et al. (2020**) there was noted it is necessary to regulate the temperature of alcoholic fermentation to values of 14 – 16 °C to obtain a high-quality organoleptic profile.

Wang et al. (2020a) detected free terpenes by using solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC–MS). Their findings indicate that the matrix effect of phenolic acids can effectively control the release and modulate the global feature of wine aromas. In another similar study **Song et al. (2020)** the determination of the total concentrations of terpene glycosides were 7.32, 3.50, and 81.27 mg.kg^{-1} in Ecolly, Cabernet Gernischet (CG), and Muscat Hamburg (MH) grapes, respectively. **Haygarov, Yoncheva and Dimitrov (2016)** determined the content of terpenes in selected grape varieties Rubin and Storgozia. The presence of terpenes was less marked and there was no significant difference in the quantity and quality of aromatic components. **Cehula et al. (2020)** presented in their study that wines with superior sensory properties did not contain higher levels of antioxidants or higher antioxidant activity. The formation of 13 volatile compounds was studied by **Lakatošová et al. (2013)**. Authentication of particular aromatic substances in typical Slovak wines and its saving in newly founded database can help to prevent wine falsification. They registered significant differences in the production of isoamyl acetate, 1-hexanol and 1-heptanol ($p \leq 0.05$).

Liu et al. (2017) noted that the major aroma components in grapes and wine include free volatile compounds and glycosidic nonvolatile compounds. Glycosidic aroma precursors are important reserves of grape and wine aroma components. Investigations concerning the chemical structures. The most used sulfur dioxide in winemaking owing to its antioxidant and antimicrobial properties. **Wang et al. (2020b)** studied to reveal the varietal characteristics of terpene glycosides (TGs) in ripe Meili grapes. They found that 49 of TGs were detected by UPLC–Q-TOF-MS. Monoterpenol pentosyl-glucosides were the main TGs, especially $C_{10}H_{18}O_2$ pentosyl-glucoside, which constituted 51.94 – 52.72% of the total concentration. **Shih et al. (2019)** found a novel application of terpene compound α-pinene for the alternative use of sulfur dioxide. They showed that α-pinene possessed an excellent antibacterial ability and could be a viable alternative for SO_2 in winemaking.

CONCLUSION

In this study, we demonstrated huge differences between measured results of content of terpenic substances in extracts of fresh berries of grape (*Vitis vinifera*) and extracts of wine. In both types of samples, we selected the typical Czech cultivar Pálava. We found significant relationships between total terpenic content *($p \leq 0.05$)*. Using uncommercial yeast could have a possible effect on the content of bioactive substances. We increased in wine volatile and terpenic substances by the used method and the results can be achieved. Also, we have once again justified the importance of wine production technology regarding fermentation.

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MILK CONSUMPTION IN CHILDHOOD AND ADULTHOOD AND ITS EFFECT ON BODY COMPOSITION

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ABSTRACT

Throughout their life, people are exposed to many different types of milk. First, it is breast milk if infants are breastfed or special formula based on cow milk with modified composition if they are not breastfed. Later in life, it is recommended that humans consume the milk of other mammals as a source of highly valuable protein, calcium, and phosphorus. This work aimed to evaluate the effects of methods and duration of feeding in infancy and consumption of milk or milk alternatives in adulthood on body composition. We used a questionnaire of 21 specific questions to obtain information on breastfeeding and milk consumption. All 84 participants (18 men, 66 women; age 23.26 ±1.36) underwent measurement of body composition, using BIA methods (InBody 720). A comparison of the information from the questionnaire with the information from the body composition measurement was made. Significant differences were observed in visceral fat area (p = 0.048) and waist-to-hip ratio (p = 0.022) according to duration of breastfeeding. Participants who were fed formula for a shorter time than 1 year (until 12 months of age) showed a higher percentage of body fat (p = 0.047). The fat percentage of milk was a significant factor for the waist-to-hip ratio (p = 0.026). Participants consuming plant-based milk alternatives showed significant differences in waist-to-hip ratio (p = 0.031) and body mass index (p = 0.015) and highly significant differences in weight (p <0.001) and fat-free mass (p <0.001). In conclusion, results show that the duration of breastfeeding may prevent the development of overweight and eventually obesity. Usage of infant formulas as an alternative to breast milk should be limited to those who are unable to breastfeed. The current consumption of milk indicates the benefits of whole milk in the diet but also shows increasing interest in the advantages of plant-based milk.

Keywords: milk; breastfeeding; plant-based milk; body composition; milk fat percentage

INTRODUCTION

From the very first day of their life, humans are introduced to milk. They are exposed to breast milk or formula as the only sustenance until 6 months of age. Breastfeeding should then continue up to 2 years of age and beyond, but children should also begin to eat adequate complementary foods as recommended by the WHO **(WHO, 2011)**. Human milk composition is dynamic and varies daily, during a feeding, based on the lactation stage, between mothers and populations. The mean macronutrient composition of mature milk is estimated to be approximately 0.9 to 1.5 g of protein, 3.2 to 3.6 g of fat, and 6.7 to 7.8 g of carbohydrates, mainly lactose (6.5g) in 100 ml. Energy is estimated to range from 65 to 70 kcal (270 to 295 kJ) in 100 ml and is highly correlated with the fat content of human milk. Macronutrient composition differs; preterm milk tending to be higher in protein and fat **(Ballard and Morrow, 2013)**. Mineral content is approximately 0.2g, represented by calcium, phosphorus, sodium, potassium, magnesium, and iron. Vitamins present in the milk include A, D, E, C, and B vitamins, but human milk is deficient in vitamin K **(Bernier, Adrian, and Vidon, 1988)**. Breastfeeding provides many positives for both infants and mothers: decreased postpartum bleeding and faster uterine involution caused by increased concentrations of oxytocin, decreased menstrual blood loss and increased child spacing attributable to lactational amenorrhea, earlier return to pre-pregnancy weight, decreased risk of breast cancer, decreased risk of ovarian cancer, and possible decreased risk of hip fractures and osteoporosis in the postmenopausal period **(American Academy of Pediatrics, 2005)**. Known effects in infants include a decrease in child morbidity and mortality; protection against diarrhea, respiratory infections, and otitis media; and a possible 68% reduction of malocclusion in children **(Victora et al., 2016)**. There are also positive effects on cognitive skills, which determine educational outcomes, and on knowledge and skill accumulation during both childhood and adult life. Longer breastfeeding duration is also associated with decreased autistic traits and decreases the risk of overweight and obesity in adulthood **(Victora et al., 2016; Boucher et al., 2017)**. However, breastfeeding for longer than 12 months or during the night increases the risk of dental caries in

deciduous teeth **(Tham et al., 2015)**. Despite all the positive effects, many infants and children do not receive optimal feeding. WHO reports that only about 44% of infants aged 0 – 6 months worldwide were exclusively breastfed throughout 2015 – 2020 **(WHO, 2020)**. Unfortunately, some mothers are not able to breastfeed. Known alternatives include human milk provided by milk banks or infant formulas. The main ingredient of an infant formula is usually cow milk, which must be modified because of its different composition than human milk **(Blanchard, Zhu and Schuck, 2013)**. The composition of infant formula is defined by The European Union Commission Directive (2006) concerning infant formula that applies to all member states **(European Commission, 2006)**. Nevertheless, formula-fed infants face a higher risk of diseases due to a lack of specific and innate immune factors present in human milk **(Stuebe, 2009)**. Later in life, it is recommended that people continue to have milk intake, most frequently cow milk, because it contains several essential nutrients **(Haas et al., 2019)**. Cow milk is proposed as a useful eatable, mainly during childhood and adolescence, when its content of calcium (125 mg per 100 ml), the protein of high biological value (3.5 g per 100 ml), phosphorus (100 mg per 100 ml), and other micronutrients promotes skeletal, muscular, and neurological development **(Blanchard, Zhu and Schuck, 2013; Visioli and Strata, 2014)**. However, its relatively high-fat content (approximately 70% SFAs; myristic and palmitic acids combined account for ~50%, the remainder are mostly short- and medium-chain FAs and oleic acid) has marked it as potentially detrimental food, especially in connection to cardiovascular health **(Visioli and Strata, 2014; Juráček et al., 2020)**. Furthermore, due to widespread health issues linked to milk consumption, such as lactose intolerance or milk allergies, and lifestyle changes, an alternative market for plant-based milk is emerging **(Chalupa-Krebzdak, Long and Bohrer, 2018)**. Plant-based milk alternatives are fluids made from ground plant matter (cereals, pseudo-cereals, legumes, oilseeds, nuts) extracted in water. Further homogenization is necessary and results in particle size distribution in the range of 5–20 μm, which imitates cow milk appearance and consistency **(Sethi, Tyagi and Anurag, 2016)**.

Scientific hypothesis

Many studies confirm and highlight the beneficial effects of breastfeeding on health in later life. This study aimed to determine whether breastfeeding in childhood affects the development of body composition and to observe differences between breastfed and formula-fed participants. We also try to refute the stigma that consuming whole milk is not suitable for maintaining optimal body weight and body fat.

MATERIAL AND METHODOLOGY

We examined the relationship between breastfeeding in childhood, milk and milk alternatives consumption, and body composition in adulthood. A questionnaire method was used to obtain information on breastfeeding in infancy and the current consumption of milk and milk alternatives. Each participant completed the questionnaire alone and anonymously. The survey involved 84 participants, 18 men (21%), and 66 women (79%). The average age of the participants was 23.26 ±1.36. The questionnaire consisted of questions on gender, age, educational level, health status, and certain anthropometric parameters, and 21 specific questions on milk consumption. Body composition data were obtained using InBody 720 (Biospace Co. Ltd., Seoul, Republic of Korea), a multi-frequency bioelectrical impedance analyzer (BIA). Measured parameters included body weight (kg), FM (fat mass, kg), FFM (fat-free mass, kg), percentage of body fat (%), VFA (visceral fat area, cm^2), WHR (waist-to-hip ratio), and BMI (body mass index). We compared the information from the questionnaire with the information from the body composition measurements. Participants undergoing measurements using BIA were healthy, without a pacemaker. None of the women were pregnant. All participants provided written consent to the bioelectrical impedance measurement.

Statistical analysis

Statistical analysis was carried out using MS Excel 2010 (Los Angeles, CA, USA) and STATISTICA Cz version 10 (**TIBCO Software Inc., Palo Alto, California, USA**). Data were expressed in **Figure 1** and **Figure 2** as a mean ± standard deviation (SD), minimum, maximum, median, and mode were calculated. Statistical comparisons between groups were made utilizing a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance levels were determined as p <0.05 (*), p <0.01 (**), and p <0.001 (***).

Table 1 Mean values of selected anthropometric parameters according to the duration of breastfeeding

Duration of breastfeeding	Anthropometric parameter						
	Weight [kg]	FFM [kg]	FM [kg]	Percentage of body fat [%]	VFA [cm^2]	WHR -	BMI [kg/m^2]
<4 months	70.66	49.94	20.72	27.46	78.60[1]	0.87[1]	24.35
4 – 6 months	65.34	49.85	18.49	23.86	62.80	0.85	22.80
6 – 12 months	67.40	50.82	16.58	24.56	67.26	0.85	23.13
>12 months	59.75	46.52	13.23	21.92	52.12[1]	0.82[1]	20.99

Note: [1]p <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Breastfeeding

We compared the participants who were breastfed as infants with those not breastfed and investigated the potential effect of breastfeeding on body composition. The differences were not significant, which could be due to the uneven distribution of participants, breastfed (n = 80), and non-breastfed (n = 4). However, breastfeeding may have a small but consistent protective effect on obesity prevention, as indicated by a meta-analysis of 9 studies with more than 69,000 subjects, concluding that breastfeeding significantly reduces the risk of developing obesity in adult humans (Arenz et al., 2004). Some studies suggest that breastfed infants tend to have lower FM when compared with formula-fed infants. When we examined the FM to weight and FM to lean body mass ratio, breastfed infants showed lower values. Formula-fed infants tend to have a lower lean body mass to body weight ratio (Pludowski et al., 2009). Breastfeeding directly from the breast appears to be the safest way for children to receive breast milk. It protects against obesity, as these children have the least chance of being overfed. In infants fed breast milk from a bottle, the risk is higher but still low, as breast milk helps to create an intestinal microbiome that is likely to help prevent obesity **(Azad et al., 2018)**. Breastfeeding protects against overweight and obesity in children by inducing lower plasma insulin levels, thereby reducing fat storage and preventing excessive early adipocyte development **(Oddy, 2012)**. Significant differences in body composition depending on the duration of breastfeeding in childhood were observed for VFA (p = 0.048; **Figure 1**) and WHR (p = 0.022). In both cases, there was a significant difference between participants breastfed for less than 4 months and those breastfed for more than 12 months. Higher mean values of VFA and WHR were present in participants breastfed for less than 4 months **(Table 1)** and therefore did not meet the WHO recommendations of min. 6 months of breastfeeding **(WHO, 2011)**. This finding agrees with the claim that infants breastfed longer (6 months or longer) have a reduced risk of developing overweight and obesity **(Victora et al., 2016)**. Long-term breastfeeding is directly related to a decreased risk of obesity **(Yan et al., 2014)**. The recorded changes in other measured parameters were not significant, but they were visible in the graphic display. Participants who were not breastfed were fed formula, which replaced breast milk. The use of infant formula increases the risk of childhood obesity due to overfeeding, and childhood obesity is often carried into adulthood **(Azad et al., 2018)**. We observed significant differences in the percentage of body fat in the group of formula-fed participants. Participants fed for only 1 year (until 12 months of age) showed higher values when compared to participants fed for 1.5 years (until 18 months of age) (26.02% to 19.16 %, respectively; p = 0.47). Exclusive breastfeeding prevents improper dietary practices, such as the early introduction of complementary foods, which could lead to unhealthy weight gain. Protein and total energy intake are higher in formula-fed infants compared to breastfed infants, which is positively associated with the development of obesity later in childhood **(WHO, 2014)**, leading to an increase in total cholesterol in adulthood **(Owen et al., 2008)**. Unfortunately, infant formula, which should be considered a specialized food that is vital for children who cannot be breastfed, is becoming a regular meal for children due to promotion and marketing **(McFadden et al., 2016)**.

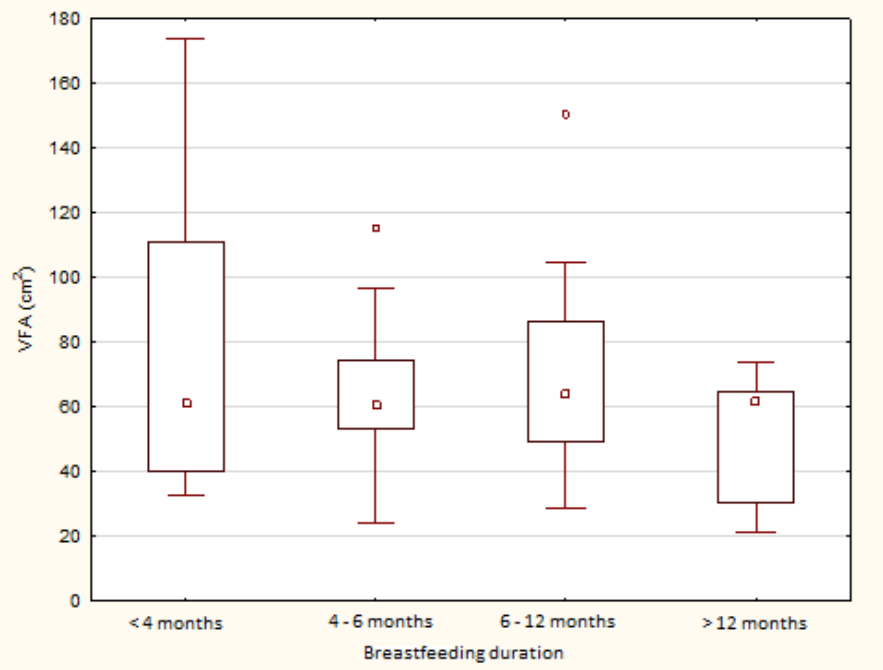

Figure 1 Differences in VFA according to duration of breastfeeding in infancy.

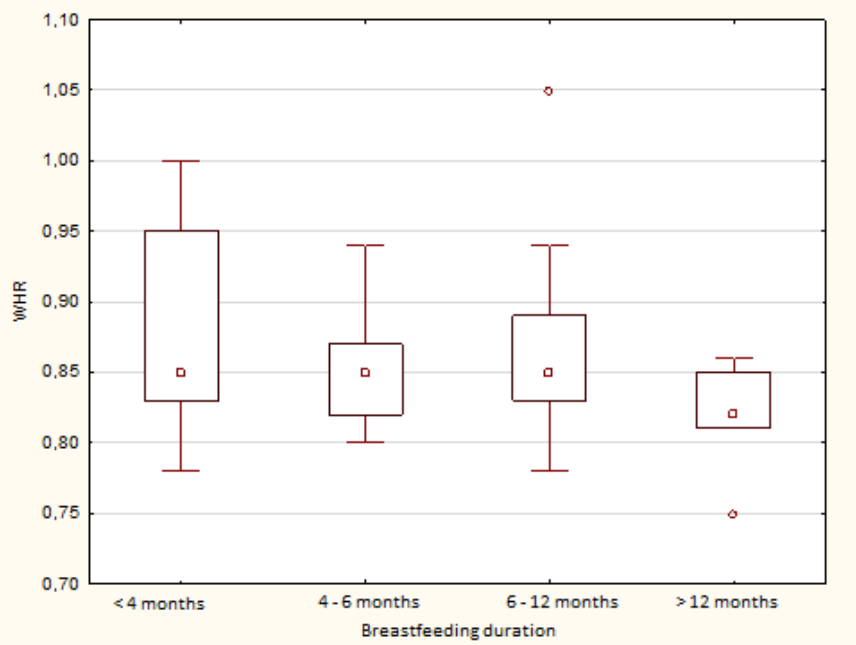

Figure 2 Differences in WHR according to duration of breastfeeding in infancy.

Current milk consumption

We investigated the effects of current milk consumption in participants on body composition. Participants were divided into 2 groups, consumers, and non-consumers of milk. We did not find any significant differences; in fact, the values of anthropometric parameters were very similar **(Table 2)**. Available scientific evidence suggests that consumption of milk and dairy products does not adversely affect body weight or body composition **(Spence, Cifelli and Miller 2011; Schwingshackl et al., 2016)**. Most of the cross-sectional and prospective studies suggest a favorable relationship between milk consumption, body weight, and body composition in children and adolescents. Also, milk is an important source of calcium, vitamin D, phosphorus, and potassium in the diet of children aged 2 to 18 **(Spence, Cifelli, and Miller 2011)**. Following the milk consumption analysis, we paid separate attention to the fat percentage in milk, according to the possibilities available on the market (fat content 0.5%; 1.5%; 3.5%). We observed a significant difference in WHR between the groups consuming milk with a fat content of 1.5% and 3.5% (0.86 and 0.83, respectively; $p = 0.026$; **Figure 2**). A higher percentage of fat in milk is inversely related to weight gain and thus is not a factor for the development of overweight and obesity **(Rautiainen et al., 2016)**. Today, many people succumb to the phenomenon of plant-based milk because of health or other reasons. Therefore, we also monitored the effect of these beverages on body composition and its parameters. The mean values of anthropometric parameters according to the consumption of plant-based milk are shown in **Table 3**. We observed statistically significant differences in the parameters of

Table 2 Mean values of selected anthropometric parameters according to current consumption of milk.

Participants	Anthropometric parameters						
	Weight [kg]	**FFM [kg]**	**FM [kg]**	**Percentage of body fat [%]**	**VFA [cm^2]**	**WHR -**	**BMI [kg/m^2]**
Consumers	67.15	50.26	16.90	24.62	67.01	0.86	23.11
Non-consumers	64.37	48.83	15.54	24.3	62.71	0.85	22.79

Table 3 Mean values of selected anthropometric parameters according to current consumption of plant-based milk.

Participants	Anthropometric parameters						
	Weight [kg]	**FFM [kg]**	**FM [kg]**	**Percentage of body fat [%]**	**VFA [cm^2]**	**WHR -**	**BMI [kg/m^2]**
Consumers	59.51[2]	44.49[2]	15.2	24.74	60.55	0.84[1]	21.86[1]
Non-consumers	77.98[2]	61.00[2]	16.98	22.3	68.75	0.89[1]	25.38[1]

Note: [1]differences at $p < 0.05$ were considered significant, [2]differences at $p < 0.001$ were considered highly significant.

weight and FFM. Mean body weight in participants consuming plant-based milk alternatives was 59.5 kg and those who did not consume plant-based milk 77.98 kg (p <0.001). Similarly, the mean FFM of consumers was 44.49 kg and of non-consumers 61.00 kg (p <0.001). Significant differences were also noted for WHR (0.84 to 0.89 respectively; p = 0.031) and BMI (21.86 vs. 25.38, respectively; p = 0.015). These differences may be due to the different nutritional composition of plant alternatives to milk, in particular their lower energy content. It is not recommended to use these beverages as substitutes for cow milk **(Singhal, Baker and Baker, 2017; Vanga and Raghavan, 2018)**, even though calcium-fortified soy beverage is comparable to cow milk in its macronutrient content **(Silva, Silva and Ribeiro, 2020)**.

CONCLUSION

We did not observe significant differences between breastfed and non-breastfed participants, but in those who were breastfed as infants, the duration of this period played a significant role. Significant differences were detected in VFA (p = 0.048) and WHR (p = 0.022) between participants breastfed for less than 4 months and breastfed for more than 12 months. This finding is consistent with the WHO recommendations and the benefits of breastfeeding. Participants that were formula-fed as infants showed a higher percentage of body fat (p = 0.047) when fed for less than 1 year (12 months). Consumption of milk did not cause significant differences in body composition. However, the fat percentage of milk was a significant factor for WHR (p = 0.026), refuting claims that whole milk is a detrimental food. Participants consuming plant-based milk alternatives showed significant differences in WHR (p = 0.031) and BMI (p = 0.015) and highly significant differences in weight (p <0.001) and FFM (p <0.001). Those differences are most likely due to the lower energy content of these beverages and various protein and fat compositions based on the raw materials used for production.

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ADHESION OF MARZIPAN PASTES BASED ON DRY DEMINERALIZED WHEY

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ABSTRACT

The results of researches of rheological and adhesion characteristics of marzipan pastes with dry demineralized whey (DDW) and glycerin are given. The positive effect of dry demineralized whey and glycerin on the characteristics of model compositions of marzipan pastes has been established. The component compatibility of DDW and almonds has been confirmed. It has been experimentally established that DDW and glycerin lead to changes in the structural state of marzipan pastes, changing the quantitative values of rheological characteristics. It is confirmed that with increasing DDW concentration, the indicators of deformation and plasticity increase. The indicators of elasticity and resilience of marzipan paste decrease, which in general leads to an increase in molding ability. The surface effect on the properties of marzipan pastes with dry demineralized whey and glycerin was carried out. The technological expediency of using glycerin in the composition of marzipan pastes with DDW to increase their plasticity and pliability while maintaining high molding properties is substantiated. The rational content of glycerin is established, which allows regulating the adhesion properties within the set limits for paste-like finishing semi-finished products from marzipan masses. The rational content of DDW and glycerin in the composition of marzipan pastes while ensuring high functional and technological properties in the application of confectionery flour on the surface is substantiated. Mathematical modeling of adhesion behavior of marzipan pastes is carried out. The adhesive properties of the pastes are mathematically substantiated and their ability to have flexibility under the action of the applied shear stress is confirmed within the established limits. Its currents are considered and substantiated. A study of moisture absorption by flour confectionery when covered with marzipan paste is provided. The wetting angle is considered and the work of adhesion is determined. Based on researches the directions of use of the developed marzipan pastes in confectionery production as finishing semi-finished products for a covering and as a layer for flour confectionery products and modeling of figured products are offered.

Keywords: marzipan paste; adhesion; whey powder; glycerin; rheological properties; modeling

INTRODUCTION

Confectionery production is an art, so there is a fleeting fashion. The quality of products significantly depends on how receptive the manufacturer is to the latest trends in the product market, how he/she knows the tastes of consumers, and their solvency. With the country's entry into a new level of market relations, the assortment policy of the food industry in general and confectionery, in particular, has changed. In the conditions of market competition more and more attention is paid to the level of aesthetics of confectionery products. The range of confectionery and semi-finished products produced in our country is diverse, constantly changing, and has about 5,000 items. Stricter consumer requirements for the taste of confectionery products with a limited raw material base and reduced quality of raw materials encourage scientists to create a new direction in the confectionery industry which is the development of compound foods based on raw materials in certain regions.

Improving technological processes is one of the main directions in the food industry. It involves the study of changes in physicochemical properties when using various methods of influencing natural raw materials used for human nutrition. Extensive opportunities in this direction open up when creating such process conditions that provide a comprehensive impact on raw materials. Therefore, the main technological directions in the development of new types of confectionery and semi-finished products are to improve the range of products for baby and dietary nutrition, increase the amount of protein, reduce carbohydrates, and especially sugar. To solve this goal, the addition of various fillers to the composition of sugar masses, which enrich the product with biologically active substances, has recently become widely used.

Modern semi-finished products for confectionery products are represented by a wide range of various creams, chocolate, marzipan, sugar, paints, toppings, cast caramel decorations, etc. A special place among the finishing confectionery semi-finished products is occupied

by marzipan mass of a mixture of raw almond kernels, apricots, or nuts. Due to the exclusivity of organoleptic properties and versatility of use in various areas of confectionery production **(Tamova, Shchikarev, and Basyuk, 2015)**, the paste has the necessary set of substances for the human body. Due to the high level of import dependence **(Dmitrieva and Makarova, 2016)**, the production of marzipan in Ukraine is limited, because 80% of the almond kernel consumed in Ukraine is of foreign production. This necessitates the search for new ingredients of domestic production, which will reduce the share of imports of dependent raw materials in the recipes of marzipan masses and reduce the cost of finished products. At the same time, it is important to preserve the typical marzipan taste, aroma, and structure of the product, as well as to increase its biological and nutritional value. Such improvement of existing technologies of marzipan pastes should be based on the use of non-traditional vegetable raw materials aimed at increasing the biological value, reducing energy consumption, improving flavor and functional and technological properties.

Analysis of model approximations of medium types

Most confectionery consists of sugar or other sweet substances (honey, xylitol, sorbitol), as well as molasses, various fruits and berries, milk, butter, cocoa beans, nut kernels, flour and other components. These are mainly sweet products that have a pleasant taste and aroma, good appearance, high nutritional value, calories and good digestibility.

In the work of **Tamova, Shchikarev and Basyuk (2015)** and **Kozlova (2014)** it is noted that the choice of process parameters involves comprehensive research to identify the nature of changes in the structure and properties of both individual components of raw materials and raw materials as a complex. Therefore, the participation of scientists in the implementation of technology is to deepen the understanding of the importance of nutrition in human life, taking into account national traditions, transformations of individual food components in the body structure, their impact on organs and systems. Preservation of valuable nutrients and BAS, the formation of habitual consumer characteristics, high bioavailability and product safety is based on the development of production technologies with the necessary physiological properties by improving recipes, methods and modes of processing raw materials, technological parameters of production.

Developed technologies of marzipan pastes **(Apet and Pashuk, 2004; Pat. No. 2015152421, 2017)** the authors aim to find and study the physiological and technological properties of non-traditional raw materials, which in terms of biologically active substances (BAS) can be attributed to the so-called functional ingredients, studying their influence on the course of preparation and formation of product quality. The recipe mainly included walnuts, hazelnuts, cashews, pecans, peanuts, chestnuts, cherry stones, apricots and pumpkin seeds. Replacement of almonds with peach or apricot pits **(Chorrna et al., 2015)** was accompanied by the name of the made persipan paste. In the works **(Chorrna et al., 2015; Tamova et al., 2015)** it is noted that the optimal quality of marzipan mass can be obtained from sweet almonds with the addition of 1 – 2% bitter. Its limitation is due to the danger of the formation of hydrocyanic acid from amygdalin.

In works (**Apet and Pashuk, 2004; Pat. No. 2015152421 (2017)** the ways of strengthening of taste and aromatic indicators of marzipan pastes are noted. Their prescription composition may include a variety of non-traditional raw materials, such as coffee meal, chicory powder, bunduk seeds. This approach gives marzipan pastes the smell of coffee and the taste of hot chocolate. At the same time, other works **(Apet, 2004; Pat. No. 20150211, 2016)** noted that the use of pear and amaranth seed powders not only enhances the taste and aromatic qualities of marzipan pastes but also improves rheological characteristics due to the high oil content. This creates the conditions for increased viscosity and adhesion stress, which improves the formation of these confectionery masses.

Reducing the caloric and glycemic content of finishing semi-finished products in their production happens by using different technological properties, origin, composition, caloric content and sweetness of sugar substitutes and sweeteners. Technologies of marzipan paste with low sugar content **(Pat. No. 20150141, 2016; Pat. No. 2016127117, 2017)** are represented by model compositions of sodium or potassium acesulfame, aspartame. The use of synthetic sweeteners indicates the lack of such compositions. Studies by the authors **(Fernandez and Santos, 2018; Zeynep and Sifa, 2014)** confirm their negative impact on human health.

The use of natural sweeteners in the technology of marzipan pastes, namely: agave syrup, erythrol, stevioside, palatinosis can significantly reduce the high caloric content **(Pat. No 20150211, 2016)**. Known technologies of marzipan masses **(Pat. No. 20150211, 2016; Pat. No. 20150141, 2016)** in which various types of flax, barley, buckwheat, corn, lentil, rice are used as fillers as well as pea flour, increase the internal forces of adhesion of the components of marzipan masses. Such fillers bind moisture better and promote the formation of a more homogeneous and plastic structure of the mixture during molding. However, despite the positive attitude, these compositions reduce the organoleptic characteristics due to the presence of a sharp cereal taste and aroma. Baby foods that are part of the diet of children need special care as objects of enrichment. Their bodies are more sensitive to non-traditional ingredients because children's metabolic systems are not yet sufficiently formed and are not able to withstand high loads, to respond adequately to them.

Dried chokeberry berries and medlar leaf powder are used as a functional additive in marzipan paste technologies. This allows not only to increase the nutritional and biological value but also to achieve the maximum possible preventive effect with a relatively small content of marzipan pastes. As an antioxidant use the drug "Cyclorar". This biologically active substance improves the nutritional value of marzipan masses and improves its molding properties.

The main disadvantage of marzipan pastes with these additives is reduced sensory characteristics. The use of additives to enrich the nutritional composition has a significant effect on changes in the color and taste of this product. As a result, there are problems with the toning of

marzipan paste and obtaining the appropriate color scheme. This makes this type of finishing semi-finished product unsuitable for confectionery coating and modeling of figured finishing semi-finished products. The raw material for the production of marzipan pastes due to biological, physical, and mechanical factors changes its properties and acquires characteristic of this type of paste. The process of production of pastes can be considered as a set of processes of change of rheological characteristics of the formed semi-finished product according to a compounding under the influence of biological, physical, and mechanical factors. On the other hand, changes in rheological characteristics affect the choice of design and modes of operation of equipment. Therefore, the complex of rheological (structural and mechanical) properties of marzipan pastes are the most important, which predict their state in a variety of technological processes and characterize the physical state, dispersion, structure, structure, and type of interaction.

It is established that methods and means of determining the rheological characteristics of different types of deformation affect product quality **(Stadnyk et al., 2019)**. In our opinion, the works by **Kravchenko et al. (2019)** give some examples of calculations of technological processes and equipment aimed at studying the physical and mechanical properties of the environment. This approach allows to improve and intensify the technological process, to develop scientifically sound methods for calculating the technology of forming marzipan pastes.

Developed technologies of marzipan and praline masses **(Sirohman and Lozova, 2008; Pat. No 2008121837, 2009)** in the prescription composition of which included milk powder, in our opinion allowed to improve consumer properties and reduce production costs. It is known that DDW is used in the manufacture of confectionery as a substitute for condensed or powdered milk in the production of creamy caramel, iris, fondant, icing, chocolate **(Kravchenko et al., 2020)**.

As a result of the analysis of literature and patent sources, the wide use of glycerin in the prescription composition of paste-like finishing semi-finished products was revealed. Glycerin in the technology of sugar pastes is used to reduce the stickiness (adhesion) of the food system, it gives softness to the pastes, improves the molding properties, makes them more pliable in the manufacturing process of finishing semi-finished products **(Gulenko, Sibileva, and Zhyvotkevych, 2013)**. This determines the relevance of research on the feasibility of using glycerin in the technology of marzipan pastes with DDW.

The creation of a new technology of marzipan pastes with DDW, the feasibility of using glycerin in their composition requires in-depth studies of rheological and sensory characteristics of finished products to justify rational technological processes, which largely determine the consumer and functional properties of marzipan pastes. The relevance of the search for new recipe ingredients for marzipan masses in the partial replacement of almond flour, the use of which can simultaneously solve several technical problems to ensure the specified organoleptic and rheological properties, is quite high.

When calculating the recipe, the main difficulties arise due to the instability of the movement of the paste, and, accordingly, shear deformations, the impact of quality indicators. At the same time, the improvement of the technological process requires the establishment of a relationship between the characteristics of the components that affect marzipan pastes in the formation of products.

Therefore, the main approach to the choice of marzipan paste formulation is to establish rheological connections, which are the most realistic for solving the problem in the formation of semi-finished products and products. Accordingly, the study of the developed marzipan pastes requires in-depth knowledge of all the intricacies of the process, its mathematical description.

Scientific hypothesis

Creation and determination in the prescription composition of marzipan pastes of rational concentration of glycerol with dry demineralized whey to ensure the specified (desired) rheological, surface characteristics, which will help to some extent in ensuring the technological feasibility of use.

MATERIAL AND METHODOLOGY

Materials

Dry demineralized whey made from cottage cheese with 90% level of demineralization under TU U 15.5-00413890-089:2014 **(Table 1)**; marzipan paste, made by traditional technology; model systems of marzipan paste with the addition of DDW in concentrations of 10 – 40% **(Table 2)**, and food glycerin TU U 10.8-40570177-001:2016: 1 – 6% **(Table 3)** of the total mass of dry components of marzipan mass (almond kernel and powdered sugar).

Table 1 Physical and chemical and organoleptic parameters of DDW.

Name of indicator	Characteristics and norm
Taste and smell	Sweet, without foreign tastes and odors
Appearance and consistency	Fine powder
Colour	Light cream
Mass fraction of dry matter, %	97.00
Mass fraction of ash, %	2.63
Mass fraction of lactose, %	75.92
Mass fraction of fat, %	1.0
Mass fraction of protein, %	15.45
The acidity is titrated	4.7
Acidity is active	5.825
Solubility index	Complete solubility

Table 2 Prescription composition of model systems of marzipan pastes, per 100 g.

Name of raw materials	Control	Samples with addition DDW, %			
		10	20	30	40
Powdered sugar	43.0	38.0	33.0	28.0	23.0
Treacle	14.0	14.0	14.0	14.0	14.0
DDW	-	10.0	20.0	30.0	40.0

Table 3 Prescription composition of model systems of marzipan pastes with MSSD, per 100 g.

Name of raw materials	Samples with the addition of glycerol, %					
	1	2	3	4	5	6
DDW 20 %						
Almond kernel	32.5	32.0	31.5	31.0	30.5	30.0
Powdered sugar	32.5	32.0	31.5	31.0	30.5	30.0
Treacle	14.0	14.0	14.0	14.0	14.0	14.0
DDW	20.0	20.0	20.0	20.0	20.0	20.0
Glycerin	1.0	2.0	3.0	4.0	5.0	6.0
DDW 30 %						
Almond kernel	27.5	27.0	26.5	26.0	25.5	25.0
Powdered sugar	27.5	27.0	26.5	26.0	25.5	25.0
Treacle	14.0	14.0	14.0	14.0	14.0	14.0
DDW	30.0	30.0	30.0	30.0	30.0	30.0
Glycerin	1.0	2.0	3.0	4.0	5.0	6.0

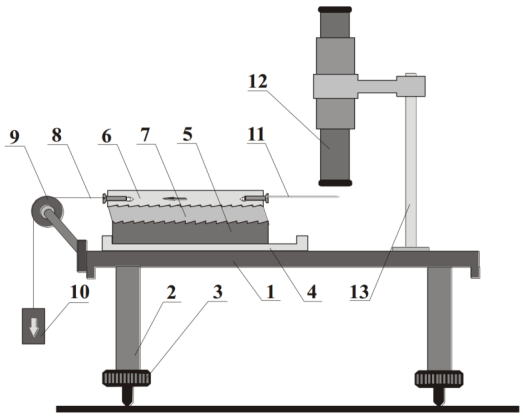

Figure 1 Installation diagram of a plane-parallel elastoplastometer Tolstoy. Note: 1 – table; 2 – support leg; 3 – adjusting screw; 4 – metal stand; 5 – metal plate; 6 – plexiglass plate; 7 – prototype; 8 – silk thread; 9 – block; 10 – cargo; 11 – observation needle; 12 – microscope; 13 – tripod.

Research methods

Sensory properties of model systems of marzipan pastes were determined according to the developed scales of organoleptic descriptors. Marzipan pastes with DDW were differentiated depending on sensory features of a consistence: marzipan pastes for a covering of confectionery; as a layer for flour and confectionery (LFC); marzipan pastes for making candies, bars, tiles; for modeling of figured products (MFP).

The study of the rheological properties of control and experimental samples was carried out using a plane-parallel plastomer of the Tolstoy modification, which is based on determining the shear deformation related to the thickness of the sample at constant stress **(Figure 1)**.

During the study of the rheological characteristics of the model systems, a fixed load was selected for all variants (65 g), provided the same temperature (+6 °C) and sample height (7 mm).

The study of the surface characteristics of the control and test samples was performed on a dynamometer connected to the MIG-1.3 measuring instrument. It allows characterizing the degree of adhesion of materials of a different structure at their surface contact. The adhesion strength was determined by the method of normal separation of the steel plate from the structured body (marzipan paste). The search for the obtained data of the results of the experimental array of the optimal mass fraction of DDW and glycerin in marzipan pastes was carried out according to known methods and methods of statistical processing using the known method of correlation and regression analysis of experimental results using differential operators qualitatively and quantitatively evaluate the characteristics in the selection of matrix ingredients**.**

Statistical analysis

This work is aimed at creating and studying the prospects of using marzipan paste based on a rational concentration of glycerol with dry demineralized whey. Estimation of the basic regularity of change of compounding structure and properties of marzipan paste is based on optimum parameters and modes at its rheological properties. The prescription composition establishes the probability of the obtained results of structure formation, necessary for the description of consumer properties. Features of the effect of glycerin and whey dry demineralized in the formation of the structure of marzipan paste and the conditions for achieving rational rheological parameters, it is possible to predict the adhesion of the paste.

A whole complex of rheological studies was carried out during the development of the prescription composition of marzipan pastes. To clarify the role of glycerol and dry

demineralized whey, the planning and formulation of computational experiments were performed to obtain the appropriate regression equations. The dependence of the structural and mechanical properties of the pastes on the content of DDW and glycerin were constructed by the method of an arbitrary experimental design.

According to the results of rheological studies, it is not recommended to increase the concentration of glycerin by over 5%. These results are confirmed by the results of sensory analysis. The zones of rational concentrations of DDW and glycerin in the composition of marzipan pastes were determined by the method of compromise solutions. Accordingly, the variables, optimization criteria, as well as the area of the definition of factors were identified.

The determined mathematical dependences of the main structural and mechanical parameters of marzipan pastes on the content of glycerin and DDW have the form:

1. by strength index:

$$Y_{1MFV}=-2.35x_1^2-1.25x_2^2-3.14x_3^2+0.13x_1x_2-$$
$$-1.52x_1x_3+0.89x_2x_3+7.27x_1+4.81x_2-6.73x_3+21.07$$

2. by extensibility index:

$$Y_{1MFV}=-1.08x_1^2-0.48x_2^2-0.26x_3^2+0.09x_1x_2-$$
$$-0.87x_1x_3+1.02x_2x_3+5.48x_1+3.48x_2-4.27x_3+7.12$$
$$Y_{2MFV}=18.6x_1^2-1.08x_2^2-0.67x_3^2+3.44x_1x_2-$$
$$-1.04x_1x_3+0.78x_2x_3+4.41x_1+5.48x_2-6.12x_3+122.12$$

3. by the ability to form index:

$$Y_{3MFV}=-0.18x_1^2-0.73x_2^2-2.61x_3^2+0.04x_1x_2-$$
$$-2.94x_1x_3+7.45x_2x_3+2.45x_1-3.57x_2+1.54x_3+2.8$$
$$Y_{3MFV}=-0.15x_1^2-0.46x_2^2-3.07x_3^2+2.01x_1x_2+$$
$$+2.01x_1x_3+6.37x_2x_3+1.76x_1-2.31x_2+1.04x_3+3.9$$

Where:

Y_{1MFV} is strength of marzipan paste *MFV*, points; Y_{2MFV} is extensibility of marzipan paste *MFV*, points; $Y_{3\ MFV}$ is ability to form, points; X1 is content of MJ and CP,%; X2 is content of glycerin,%; X3 is MSDS content,%.

By integration, the functions were obtained and the areas of optimal parameters of glycerol and DDW content in marzipan pastes were determined **(Table 4)**.

The optimal values are selected by rounding the optimized values within the compromise areas, to facilitate the dosing of the components of the formulation of marzipan pastes in the production environment.

Thus, the results of rheological and sensory studies confirmed the possibility of adding DDW to the prescription composition of marzipan pastes at a concentration of 10 – 20%, which allows the improvement of their sensory, technological, and functional properties.

RESULTS AND DISCUSSION

Meeting the needs for high-quality food is one of the main social and economic problems of today. Quality issues **(The European Commission, 2005; DSTU 3355-96, 1996; DSTU ISO 9001, 2015)** in particular the development of new products at domestic food companies, are now receiving increasing attention. Consumption or maintenance of a balanced and nutritious diet is ensured by the implementation of national programs to improve food quality and an integrated food safety system. That is why the problems of ensuring the safety and quality of products are becoming increasingly important for the food industry of Ukraine. This is facilitated by the country's transition to new political and economic relations with the European Union.

An in-depth analysis of rheological and sensory studies of newly developed formulations allows for their further use. Therefore, based on our determined optimal values of marzipan paste LFC, we analyze the effect on the strength of adhesion. The theoretical estimate of adhesion is currently very approximate, due not only to the imperfection of the equations used to calculate the strength of intermolecular bonds but also to the fact that it is impossible to estimate the actual number of bonds per unit area. Also, it is difficult to estimate the true contact area, which is always much larger during visual observation, due to the presence of roughness in the surface layer.

The study of the application of marzipan pastes on confectionery semi-finished products allows us to determine and justify their rationale prescription composition. Adhesion according to **(Adamson, 1976; Rydil, 1936)** as a surface phenomenon is associated with rheological parameters and characterizes the volumetric properties of marzipan pastes. It occurs at the boundary between two phases of heterogeneous condensed bodies: marzipan paste is one phase, the contact surface is the second phase, which causes adhesion.

The volumetric properties of the paste determine the area of contact of the two bodies, which affects the amount of adhesion and its consequence, which characterizes the state of the surface after removal of the adhering mass of the paste.

Adhesion has concomitant phenomena that characterize the bulk properties of food masses and significantly affect the adhesion interaction of the components of marzipan pastes **(Kravchenko et al., 2019)**. The influence of volumetric characteristics of food masses on surface properties can be traced by considering the ratio of adhesion and cohesion **(Stadnyk et al., 2019)**. In the case of adhesion, there is a limit of phase distribution, for cohesion such a limit is absent **(Wake, 1976)**. Cohesion is the body's resistance to destruction associated with overcoming the forces of interaction between atoms and molecules at the interface and means bonding within marzipan pastes, ie within a single phase.

Table 4 The range of optimal parameters of glycerol and MSDS content in marzipan pastes.

Model compositions of marzipan pastes	X_1(glycerin), %			X_2(DDW) %		
	$X_{1\ min}$	$X_{1\ min}$	Optimal value	$X_{2\ min}$	$X_{2\ max}$	Optimal value
Marzipan paste (LFC)	4.8	5.3	5.0	27.0	33.7	30.0
Marzipan paste (MFP)	4.4	4.5	5.0	18.2	22.4	20.0

In the process of applying effort to the marzipan paste when applied to the workpiece is the fractional interaction of their surfaces. The nature of the mass flow of marzipan paste in the form of different profiles is determined by the structural and mechanical properties and the strength of interaction (sticking) with the contact surfaces. Therefore, the amount of adhesion, in this case, is characterized by the force of separation, the specific work of separation relative to the unit area, the contact time to change the bonding conditions between the substrate and the adhesive under load. Therefore, the maximum increase in the forces of interaction of the paste with the contact area of the workpiece is characteristic. Violation of these relationships leads to the production of low-quality products and reduces the efficiency of the process. The phenomenon of the interaction of the above-mentioned bodies is rather little studied, and the nature of adhesion requires research. In works (**Zimon, 1974)** the nature of adhesion is often explained by diffusion and electrical theory. Adhesion is always the result of the intermolecular interaction of surfaces different in nature **(Deryagin, 1978; Zimon, 1974)**.

In the food industry, there are many processes in which the forces of friction and adhesion interact simultaneously. These phenomena occur when the relative displacement of the contact surfaces of the two bodies. In researches by (**Deryagin 1978),** simultaneous interaction of force of friction and adhesion is considered and their connection is established. The law expresses the proportionality of friction forces to a normal load. Some researchers as (**Souheng, 1982; Lee, 1979)** believe that the only cause of external friction is the forces of attraction between the surfaces of bodies. Chemical adhesion may occur during the adhesion of liquid and elastic and plastic food masses **(Berlin and Basik, 1974)**.

In our process, a liquid meniscus appears between the contacting phase parts. In this case, it seems to tighten the particle and the surface. The amount of adhesion will be determined by capillary force:

$$F_k = 4\pi\sigma_T r \quad (1)$$

Where:
σpn is the surface tension of the liquid condensed between the contacting bodies.

According to (**Vakula and Prytinin, 1984)** there is a spreading of a thin layer of liquid that is on a body surface.

If the “adhesion” or “adhesion force” is evaluated by the dependence of M. Stefan and Gorbatov, then such a process takes place in time, ie adhesion is evaluated as a process that must be performed to separate the samples **(Adamson and Gast, 1997; Rydil, 1936)**. Distance differences can characterize the phenomenon of cohesion. In this case, the pressing of the two bodies is a capillary interaction:

$$P = 2\alpha F \cos\beta / h = 2\alpha F_k / h \quad (2)$$

Where:

α is the coefficient of surface tension, N.m^{-1}; $cos\beta$ characterizes surface wetting; α 'is the coefficient of surface tension at full wetting, N.m^{-1}; h is the thickness of the layer of marzipan paste, m.

Assuming that the force applied from the outside to the paste increases linearly with time:

$$P = \omega c * \tau,$$

Where:
ωc is the rate of increase of force, N.s^{-1}; τ is an hour, s.

Assuming that the contact area also varies according to the linear law, we obtain:

$$S = S_0 \cdot C\tau \quad (3)$$

Where:
C is the coefficient of proportionality, which depends on the properties of the product and characterizes the rate of decrease in the area; *S0* is the current value of the area of actual contact, m2.

Substituting (2 and 3) into the dependence of M. Stefan and Gorbatov, taking into account that at the moment of separation his time is obtained:

$$P_0 = \frac{2\alpha}{h} \cdot \frac{\omega_c}{\omega_c + \frac{2\alpha \cdot C}{h}} \quad (4)$$

For convenience, the equation is represented as (5)

$$\frac{1}{P_0} = \frac{h}{2\alpha} \cdot \frac{C}{\omega_c} \quad (5)$$

Since it is assumed that the increase in force and the change in the contact area are linearly dependent on time, we can assume that $C/\omega c$ is a constant value of A, ie:

$$\frac{1}{P_0} = \frac{h}{2\alpha} + A \quad (6)$$

The amount of adhesion is directly proportional to the tension surface of the marzipan paste and inversely proportional to the thickness of its layer. This dependence (6) can occur at a small thickness of the product layer.

Using such thermodynamic concepts as free surface energy and surface tension, we can describe some stages of the adhesive interaction of the process of applying marzipan paste on the surface of the flour confectionery, **Figure 2** (wetting the adhesive surface of the substrate). Despite the extreme importance, the processes of wetting and adhesion are still not clear enough, their study continues in all developed countries. Researchers are now particularly interested in the kinetics of wetting, nonequilibrium wetting, and other aspects of wetting processes.

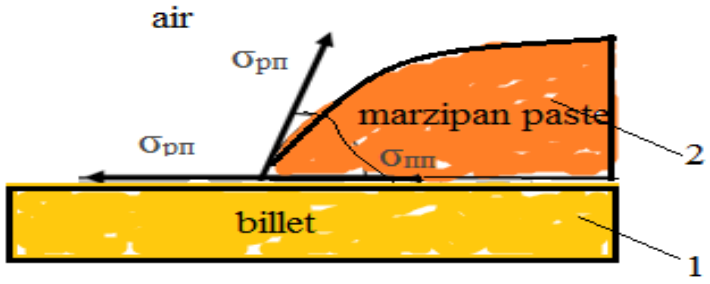

Figure 2 Equilibrium of forces affecting the angle of contact of marzipan paste 2 with the surface of the workpiece 1.

We consider the nature of their interaction as a process that occurs on the verge of contact of three phases. Wetting is also a manifestation of molecular forces, a manifestation of the affinity of the adhesive to the substrate **(Adamson and Gast, 1997; Rydil, 1936)**.

The paper by **(Shanina et al. 2019)** used the method of studying the surface tension at the interface between the phases, which allowed us to obtain very reliable research data. The study of wetting of different substrates is of interest in that it allows to identify the affinity of the adhesive to the substrate, to compare the molecular forces acting in different systems of adhesive which is the substrate.

All known methods and devices for determining surface tension are considered and analyzed in the works by **(Souheng 1982); Lee 1979); Adamson 1976); Rydil 1936)**. Moreover, in the work of the authors **(Fisher, 1979)** the technical means that allow automating the measurement process are described.

When applying marzipan paste on a hard surface of the workpiece, as noted earlier, there is a process of involuntary increase in the contact area, there is wetting. A layer of paste applied to the surface of a solid confectionery is a special physical object, the shape and structure of which is determined by the prescription composition.

In the work by **(Berlin and Basik, 1974)** it is noted that the application of a viscous liquid with its fluidity depends on the environmental conditions and the properties of the surface on which it is applied. Our offered marzipan pastes should ensure their smooth application (wetting) of the substrate surface, as well as interfacial contact between the adhesive and the substrate and interfacial or adsorption interaction at the interface of the two phases. To achieve a good application of the paste (wetting) with good adhesion, it is necessary that the surface tension of the adhesive was greater than the surface tension of the substrate. The wetting phenomenon is associated with the ratio of surface tensions (σ) of the adhesive and the substrate.

Conditions for applying a paste (wetting) of a hard surface

If before the collision with the surface the paste layer had a surface *Sk*, and the surface of the solid workpiece was *St*, then in equilibrium, when the paste layer forms a drop of a certain shape on the body surface, the surface area of their collision will be *Sc*, and the paste surface area *Sp*. The total free surface energy at the initial moment is:

$$E = S_k\sigma_n + S_T\sigma_T \qquad (7)$$

Finally, after reaching equilibrium, the total free surface energy will be:

$$E_2 = S_n\sigma_n + S_{TP}\sigma_{TP}$$

It is known that a necessary condition for the spontaneous process of applying the paste is that there is a decline in free surface energy **(Berlin and Basik, 1974)**. In our case, when stopping the application (the action of shear stress), the decline occurs quite quickly:

$$\Delta E = (E_1 - E_2) \prec 0 \qquad (8)$$

For this condition of applying the paste, the inequality is valid:

$$\frac{\sigma_T - \sigma_{Tn}}{\sigma_n} \succ \frac{S_n - S_к}{S_{тп}} \qquad (9)$$

It follows that at σт> σтп there is an increase in the surface contact of the paste with the medium (Sp> Sk). Thus, the application (wetting) is thermodynamically possible under the condition σт> σтп.

The equilibrium of the paste layer on the surface of a solid body (excluding the surface roughness and the action of gravity) is subject to the Jung equation from which it follows:

$$\sigma_T = \sigma_{nT} + \sigma_n cos\theta$$

Where:

Θ is the edge angle or wetting angle. From which it follows:

$$\cos\Theta = \frac{\sigma_T - \sigma_{Tп}}{\sigma_п}$$

Analysis of the state of equilibrium in the ternary system (solid-liquid – gas (air)) leads to the dependence of the form:

$$\sigma_T = \sigma_п \quad (10) \quad \sigma_т = \sigma_п \frac{1+\cos\theta}{1-\cos\theta} \frac{\sigma_T - \sigma_{Tn}}{\sigma_n} \succ \frac{S_n - S_к}{S_{тп}} \qquad (10)$$

Interfacial surface tension for two media is determined by Antonov's rule, according to which the surface tension is equal to the difference of surface tensions of each of the phases separately **(Adamson and Gast, 1997; Rydil, 1936)**.

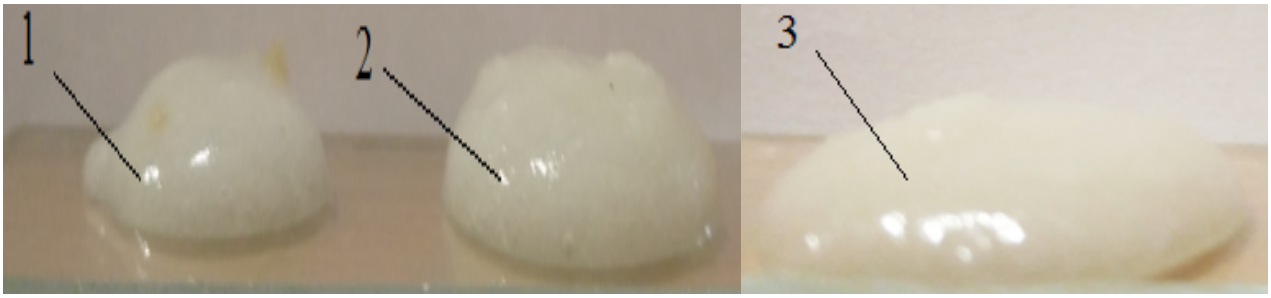

Figure 3 Blurring of marzipan paste.
Note: 1 – beginning of application, 2 – aging time 3 minutes; 3 – standing time 7 minutes.

Assuming this assumption for solid-liquid systems, we can write

$$\sigma_{тп} = |\sigma_т - \sigma_п| = |\sigma_п - \sigma_т|$$

Then, using Jung's rule **(Adamson and Gast, 1997; Rydil, 1936)** it is possible to receive a simple expression for the value of surface energy of a firm body:

$$\sigma_T = |\sigma_n - \sigma_T| + \sigma_n cos\theta \quad (11)$$

$$\sigma_T + \sigma_T = \sigma_n + \sigma_n cos\theta \quad (12)$$

$$2\sigma_T = \sigma_n(1 + cos\theta) \quad (13)$$

$$\sigma_T = \frac{1}{2}\sigma_n(1 + cos\theta) \quad (14)$$

It follows that the determination of the magnitude of the surface energy of a solid is possible by measuring the marginal wetting angle, ie the layer of paste **(Figure 3)**.

Determination of wetting angle of marzipan pastes

The study of the contact angle θ was determined by the method of dynamic measurement, provided that the phase boundary moves and there is a change in the contact angle over time is the method of changing the volume of the drop **(Figure 3)**. Among these processes, sorption and dissolution in the boundary layer of the two contacting phases promotes the diffusion of atoms (molecules) through the layer of confectionery and paste to desorb and release moisture.

The method of operative determination of the wetting angle was used to calculate the values of the derivative at the point of contact of the marzipan paste with the solid surface of the sponge blank. The surface of the marzipan paste is not described by simple analytical expressions. For an analytical description of the profile line of such a surface, you can use an approximation polynomial of the form:

$$y = a_0 + a_1 x + a_2 x^2 + a_3 x^3$$

Where:

x and y are, respectively, the vertical and horizontal coordinates of the point on the surface of the marzipan paste;

$a_0, a_1, a_2 i a_3$ are polynomial coefficients.

The value of the angle, in this case, can be found by determining the derivative at the point of three-phase contact:

$$y' = tg\theta = a_1 + 2a_2 x + 3a_3 x^2$$

Obviously, at $\sigma_т < \sigma_{тп}$ and $\cos\Theta < 0$, ie when the marzipan paste does not stick (wet) to the surface, the edge angle Θ must be less than 90 °. If the wetting angle is more than 90 °, partial wetting occurs. At Θ = 0, when the edge angle is not formed, there is a complete wetting, or spreading, which in our conditions does not occur.

$$\sigma_т > \sigma_п + \sigma_{тп}. \quad (15)$$

Thus, the wetting edge angle Θ or cos Θ is a measure of the application of marzipan paste. In the manifestation of physicochemical hysteresis, the wetting angles of the marzipan paste depend on the contact time of the phases during application to the surface of the workpiece. The surface of the workpiece marzipan paste covers completely, without the formation of voids. Therefore, the shape of the drop corresponds to a minimum of free energy. In our case we use the formula:

$$\theta_z = |arctg(a_1 + 2a_2 x + 3a_3 x^2)|$$

According to our research and data processing, the contact equation of applying the paste at an angle will be:

$$y' = -0.677 + 1.641x + 0.013x^2 + 2.454 \cdot 10^{-3} x^3$$

The equation for determining the angle will be:

$$\theta_z = |arctg(1.641 + 0.013x + 2.454 \cdot 10^{-4} x^2)|$$

Therefore, to achieve high adhesion, the surface tension of the paste must be of great importance.

It is also necessary that the surface tension of the solid confectionery was greater than the surface tension of the paste in contact with it.

Under such conditions, the application of adhesive on the surface of the substrate will be provided:

$$\Sigma \text{ substrate} > \sigma \text{ adhesive} \quad (16)$$

High-quality application of marzipan paste on flour confectionery allows moisture to penetrate some of its layers.

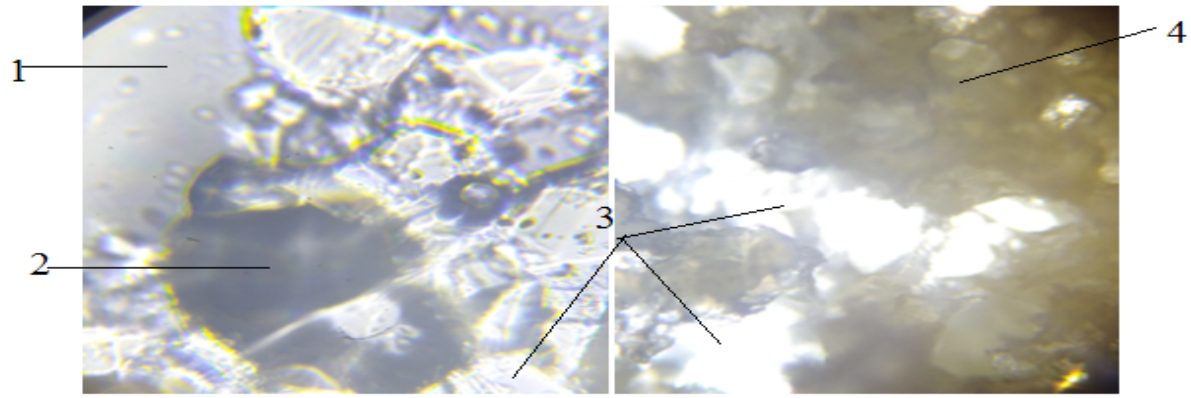

Figure 4 Photos of penetration of moisture of marzipan paste into a firm surface of flour preparation.
Note: 1 – marzipan; 2 – workpiece; 3 – air cavities in the workpiece; 4 – moisture penetration of the paste.

We made a section of applying the paste before and after 30 s of its contact. Photographs of the section under the microscope are presented in **Figure 4**.

According to the developed recipe, to determine the rate of penetration of moisture of marzipan pastes into the surface of the confectionery was used by weight method of research at a temperature of 25 °C.

The amount of moisture that penetrated through the surface of the confectionery was periodically weighed with an error of not more than 0.001 g. The change in the mass of the workpiece determines the amount of moisture that has penetrated through the test material. The experimentally established dependences of the change in the mass of the experimental samples in the time measured in min are shown in **Figure 5**.

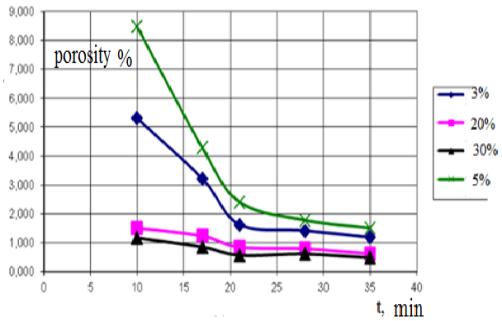

Figure 5 The rate of moisture permeability of marzipan pastes in the workpiece surface.

From the graphs shown in **Figure 5**, it follows that the absorbency of the sample coated with marzipan paste with a content of 20% and 30% DDW remained stable for 15 – 20 min from the beginning of the experiment. The less-dense molecular structure of marzipan paste with glycerin causes a greater ability to pass moisture into the workpiece. It may be desirable for the active application and coating of confectionery semi-finished products.

Calculation of the process

The effectiveness of this modification can be assessed by calculating the work of adhesion (Wa), which is determined **(Zimon, 1974)** by the ratio of surface energies of the adhesive, substrate, as well as interfacial energy:

$$W_a = \sigma_{ng} + \sigma_{Tg} - \sigma_{Tn} \quad (17)$$

Where:

σ_{ng} is the surface tension of the paste (adhesive) on the border with gas (Air), MJ.m^{-2}; σ_{Tg} is the surface tension of solid body (substrate) at the boundary with gas (air), MJ.m^{-2}; σ_{Tn} is interphase surface tension, MJ.m^{-2}.

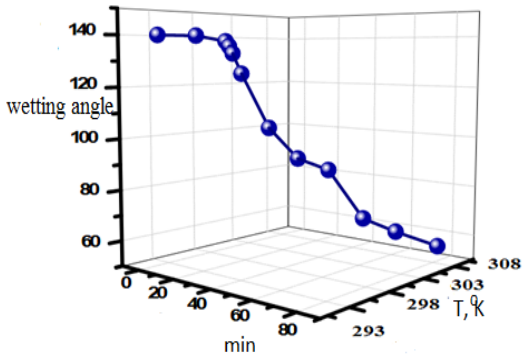

Figure 6 Dependences of the wetting angle on the ambient temperature and holding time for the pasta-confectionery system.

Temperature and time dependences of wetting are investigated. **Figure 6** presents the results of the experiments. It is shown that in the temperature range 293 – 308 the conditions for wetting the workpiece surface (θ >90 deg) are not created. The dependence of the wetting angle in the system of marzipan paste (sponge cake) on the temperature is nonlinear, which may indicate a chemical interaction. The calculation of temperature changes showed that the probability of such a process significantly depends on the temperature of the experiment. The process of flow at the adhesion boundaries at the contact of the paste with the workpiece is best at temperatures that are present in the production premises: 20 – 250 °C, which is an improvement in wetting in the system.

σ_{ng} is the surface tension of the paste (adhesive) on the border with gas (Air), MJ.m^{-2}; σ_{Tg} is the surface tension of solid body (substrate) at the boundary with gas (air), MJ.m^{-2}; σ_{Tn} is interphase surface tension, MJ.m^{-2}.

Taking into account Young's equation, the work of adhesion can be determined by the formula, which uses the values available for experimental determination:

$$W_a = \sigma_{ng}(1 + cos\theta) \quad (18)$$

Taking into account the interfacial interaction, a more accurate value of the adhesion can be determined by the formula:

$$W_a = \sigma_{ng}(2 + b \cdot \sigma_{kr}) - b \cdot \sigma_{kr}^2 \quad (19)$$

Where:

b is the coefficient of proportionality equal to the tangent of the angle of inclination, depending on cosθ = f (σn) to the abscissa.

Σkr is the critical value of the surface tension of the adhesive, which provides complete wetting, MJ.m^{-2} (equal to the value of the surface tension of the substrate).

Dependence (19) is the equation of the parabola, the vertex of which is at the following value of the surface tension of the adhesive:

$$\sigma_{ng} = \frac{1}{b} + 0.5\sigma_{kr} \quad (20)$$

The maximum adhesion work is determined by the dependence of the type:

$$W_{a\max} = \frac{1}{b} + \sigma_{kr} + 0.25b\sigma_{kr}^2 \quad (21)$$

Figure 7 shows the dependence of the adhesion on the surface tension of marzipan paste with different compositions.

The performed calculations and constructed graphical dependences testify to an increase of work of adhesion and

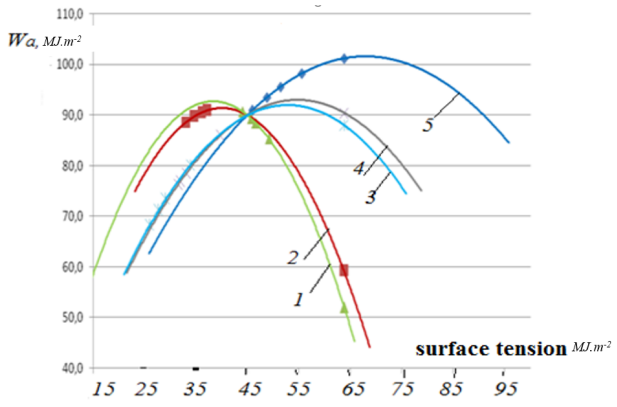

Figure 7 The dependence of the adhesion on the surface tension of marzipan paste according to the recipe.
Note: 1,2 – glycerol 3% and MSDS 20%, 3,4 – glycerin 4% and DDW 25%, 5 – glycerin 5% and DDW 30%.

the use of paste with DDW of 20% and 30% and glycerin to 5%. The most effective indicators of marzipan **pastes (Figure 7 item 5)** with 30% DDW and 5% glycerin. Therefore, this recipe has the most significant effect compared to other recipes. This is due to the quality distribution of marzipan paste and its adhesive properties. Partial replacement of almond flour with DDW in the prescription composition of marzipan pastes leads to changes in the structural state and quantitative values of rheological and sensory characteristics.

Indicators of the total deformation with increasing concentration of glycerol do not increase in direct proportion and depend on the mass fraction of DDW in the composition of marzipan pastes. Accordingly, at a concentration of DDW of 20%, the indicators of total deformation increase depending on the glycerol content in 1.0 – 1.2 times, at a concentration of DDW of 30% increase in 1.0 – 1.3 times, respectively. Irreversible deformation at a concentration of DDW of 20% is constant and does not depend on the concentration of glycerol. At a concentration of DDW of 30% with increasing concentration of glycerol, the reverse deformation increases by 1.5 times. The inverse deformation increases in direct proportion to the total deformation

CONCLUSION

The current direction of improving the preparation of marzipan pastes is and remains the search for promising sources of raw materials and the establishment of rational methods of their introduction, which will create optimal conditions with given biotechnological properties and establish mechanisms and factors of their formation. From the given mathematical analysis the compounding and selection of parameters of preparation of marzipan pastes are substantiated. Thus, in the process of formation, an increase in the strength of adhesion with an increase in the concentration of DDW, which is confirmed by sensory studies, a significant increase in stickiness. The surface properties of the pastes depending on the time of contact with the adhesive and air confirmed the fact of lengthening the working time, which is a very important factor in the modeling of shaped finishing semi-finished products made by hand. Based on the carried out sensory and rheological characteristics the directions of technological use of marzipan pastes which basis is based on structural properties are offered. Marzipan paste PKV is intended for a covering and as a layer for flour and confectionery products as the main indicator of consistency is extensibility. The best indicators were found at the concentration of DDW of 25% and glycerol of 4.5%. For marzipan paste, LFC used for the manufacture of candies, bars, tiles, as well as modeling of shaped products, the main criterion for the characteristics of the consistency is the forming ability of the paste. The best indicators are established at a concentration of DDW of 30%.

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AROMA MARKETING AS A TOOL TO INCREASE TURNOVER IN A CHOSEN BUSINESS ENTITY

Jakub Berčík, Roderik Virágh, Zdenka Kádeková, Tatiana Duchoňová

ABSTRACT

The paper deals with the evaluation of the effectiveness of the application of aroma marketing describing a few possibilities of using aromatization in practice. Nowadays, many sophisticated tools are used in marketing and consumer behavior, such as sensory marketing and sensory perception. The following is the term of marketing communication and its division into the above-the-line and below-the-line communication, sales promotion, and in-store communication. The paper also focused on the new trends in the place of sale and aroma marketing. The subject of the practical part is the use of the aroma in the food store. An important part consists of a characteristic of the alliance, questionnaire survey, comparison of achieved sales volume and sales before placing the aroma diffuser, and while it was placed in the grocery store. The article aims to find out how the coffee aroma in the store influenced consumer decision-making and stimulated them to impulsive purchase and consequent influence on the company turnover. Concerning the results of our observation and questionnaire survey, we formulate suggestions and recommendations for the business operation and practice. The whole research was made in the grocery store "Môj obchod".

Keywords: aroma; aroma marketing; marketing communication; Môj obchod; sensory marketing

INTRODUCTION

The use of scent in retail and sales, in general, is astonishing. Scent's main role is to make the customer feel comfortable, happy and put them at ease so they will spend more time in the store, spend more money and ultimately make them more likely to return. The type of scent that is used depends on what is being sold, who it is being sold to etc **(American Marketing Association, 2020)**.

Nowadays consumer behavior has an increasing role in launching products on the market **(Džupina, Hodinková and Kiková, 2016)**. Although the brand has a huge impact on the consumer's purchasing decision, it is closely related to the product placement in the stores because the final purchase decision by the consumer is made in the store or point of sale **(Košičiarová, 2013)**. The fact is that the environment is the most powerful influence when making the final purchase decision **(Doric, Primorac and Kozina, 2016)**.

Aroma marketing dynamically increases commercial results, creates the setting for a pleasant stay in private and public areas, and enhances consumer response, loyalty, and trust in the brand **(E2 Aroma, 2020)**.

The human senses act as an extraordinary source of information processing and generating **(Krishna and Schwarz, 2014)**. They must be perceived to help understand consumer processes, in which individual behavior and decision-making are important factors. Sensory marketing is an innovative marketing strategy to stimulate the customer's relationship with the brand, which promotes a lasting emotional connection that optimizes brand loyalty **(Hussain, 2019)**.

Appealing to customers' sense of smell creates sales opportunities by putting them in the buying mood **(Abassi, 2016)**. According to **Pajonk and Plevová (2015)**, aroma marketing is a series of events in which aromas can encourage customers to buy goods and services and increase the activity of employees. Several authors deal with the placement of products in the store and their impact on the customer **(Zajác et al., 2015; Kozelová et al., 2013)**.

The set up of the store is perceived by the consumer through all his sense organs. This form of perception can be defined as the process by which customers organize, obtain, and select the necessary information from the environment **(Berčík et al., 2016)**.

People constantly use the senses to create and renew impressions of the stimuli around them, and these impressions are stored and processed, leading to the creation of the meaning of the stimuli. This can further help them to make decisions. It is considered important to understand the behavior of the senses during consumer decision making **(Krishna and Schwarz, 2014)**. The challenge for marketers is to explore, understand and

stimulate all five senses of consumers, which can lead to a shift in the behavior of consumers who have a direct impact on turnover, profits, and market share **(Hussain, 2019)**.

The senses help to understand the things going on around us by recalling the information from the memories. Our sensor systems constantly encode, acquire, and reconstruct information. Our social background and cultural differences affect the way our senses connect with our memories. This type of information is important for organizations in developing their marketing strategies for sensory challenges in brand communication **(Nghiêm-Phú, 2017)**.

Erdil explained the application mechanism of sensory marketing: it creates stimulation based on external environmental factors that can appeal to the senses of consumers. These external environmental factors affect not only the emotions of consumers, but also the evaluation of products or brands, so these factors may ultimately affect the intentions and behavior of customers when shopping **(Erdil, 2015)**.

When selling, as well as when buying, all 5 senses are needed – sight, hearing, smell, taste, and touch **(Krishna and Schwarz, 2014)**.

Colors have also a meaning, but in any country or culture, it can have an opposite meaning. Some countries can be very sensitive to the color chosen from a marketing point of view, so it is necessary to choose the color according to the country of operation **(Horská, Palúchová and Gálová, 2018)**.

Zachar (2011) emphasizes that hearing is one of the most used senses, although its potential in marketing communication is not so much used. Approximately 12% of the human perception of the environment is occupied by hearing, another 50% are unknowingly accepted.

Over time, fragrances become among the basic elements of the image of the work environment. Nowadays, it is not considered exceptional that companies or various institutions have a specific scent that makes them recognizable. Fragrances, in addition to inducing a feeling of freshness and purity, also perform another function and that is to induce a feeling of holidays, customs, and traditions **(Pajonk and Plevová, 2015)**.

Although the receptors sensitive to a particular chemical are scattered haphazardly in the nose, their axons find their way to the same target cells in the olfactory bulb, in a way that chemicals of similar smell excite neighboring areas, and chemicals of different smell excite more separated areas **(Uchida et al., 2000)**.

The olfactory bulb sends axons to the olfactory area of the cerebral cortex. A complex substance, such as food, activates a scattered population of cells **(Lin, Shea and Katz, 2006; Rennaker et al., 2007)**.

Hultén, Broweus and van Dijk (2009) consider taste to be one of the most obvious senses that one perceives. On the tongue and even in the throat, a person has taste buds, which are otherwise called taste receptors, through which we can recognize individual tastes. The combination of taste and other senses creates a taste experience, such as smell and taste, sound, and taste.

Lišková (2014) in her publication states that touch is the oldest of all five senses. It is unique in the fact that unlike other human senses, which have a specific place on the human body – eyes, ears, mouth, nose, only the touch is perceived by the skin all over the human body. The hands and tongue are the most sensitive to touch, the other parts prove a different degree of sensitivity. For a trader to choose a business environment, it is necessary also to think about the touch, because both are closely related. In our opinion, smell is the most important sense that affects the customer when buying and therefore we pay more attention to it in the submitted paper.

Erenkol and Merve (2015) emphasize that almost 75% of the feelings that occur during a day are regulated by odors. Aroma directly affects the limbic system, which controls feelings and memory segments in the brain. The odor is of emotional significance to humans, on average one can recognize up to 10,000 scents, and 65% of the fragrances that man has felt in the past are stored in the brain for up to one year **(Krishna, 2010)**.

The pleasant smell released into the air keeps the buyer in the store longer, besides it has a positive effect on his desire for a product or service and at the same time increases his willingness to invest more money when buying **(Paluchová, Berčík and Neomániová, 2016)**.

Smells and odors characterize products, determine their specificity and uniqueness. The products usually have their characteristic odor, based on which they can be distinguished from others. According to different scents, an image is created not only of individual products but also of employees, for example. Managers or traders often suffer from the appropriate choice of perfumes, which they consider to be part of their expression and overall image **(Pajonk and Plevová, 2015)**.

According to **Štibinger (2012)**, each person also interprets the aroma in their unique way. It is proven that two people will never feel the same, even though it is the same chemical substance. It is also remarkable that 80% of what we perceive as taste passes through the olfactory sense.

The use of aromas can be included in the basic communication functions of the seller – customer **(Vysekalová, 2012; Abe, 2005)**.

According to **Lindström (2009)**, it can be said that in the connection between research and the use of aroma to improve the economic situation, a new division was born, which we classify under sensory marketing and even higher under neuromarketing creating a new term "Scent marketing" **(Štefániková et al., 2020)**.

Jurášková and Horňák (2012) define that scent marketing is perceived as a form of sensory marketing that focuses on activating one particular sense, which is the smell. To have a positive effect, it uses aromatizers or diffusers (which release odors into the environment) to its advantage. The meaning of this marketing is considered not only to spray a pleasant scent into the sales area but also to achieve the best possible results by choosing the right essence, which is applied at the right time to the right place.

Aroma marketing can also be included in the field of digital technologies, as they are used in aroma diffuser units **(Frey, 2017)**.

Scientific hypothesis

We have set the following research assumptions for our research:

Assumption 1: We assume that the customers will perceive individual factors differently before using the aroma diffuser.

Assumption 2: We assume that the feelings of the store atmosphere before and after using the aroma diffuser are different.
Assumption 3: We assume there is a difference between the impression of smell in the store before and after using the aroma diffuser.
Assumption 4: We assume that the significance of the smell after using the aroma diffuser is more important.
Hypotheses H_0 and H_1 were created for each assumption, which was verified by using the statistical tests. Hypothesis H_0 states that there is no difference between the indicators, hypothesis H_1 states that there is a difference between the indicators. The results of the hypotheses are given in the results of the work.

MATERIAL AND METHODOLOGY

Before researching real conditions, the influence of selected coffee aromas was tested in laboratory conditions using electroencephalography (16-channel mobile device from Emotiv EPOC). The subject of the test was four coffee aromas, the aim of which was to determine their effect on the emotional response (valence) (Table 1).

We decided to test the difference in emotions (valence) also by the statistical test which proved the significant differences in the emotions of participants tested the aroma of "Coffee" flavor and "Coffee House", "Cappuccino" as well as "Coffee and Cake". Conversely, differences have not been confirmed between the aromas of "Coffee House", "Cappuccino" and "Coffee and Cake", which may be since these are sweeter aromas (Table 2).

The subject of the testing were aromas: "Coffee House", "Cappucino", "Coffee and Cake"and "Coffe" for which we assumed the best influence on the sale of "TO GO" goods. Conscious evaluations were performed on a scale of 1 to 10, (where 10 meant the best evaluation) immediately after smelling the given sample.

METRO Cash & Carry offers marketing cooperation, which is based on a unified identity for small sellers with mixed goods, through the store chain called "Môj obchod". This project was established in 2012 and already in September 2013, the number of participating stores climbed to 100. There are currently up to 530 established "Môj obchod" stores in Slovakia and a network of stores operating abroad, in the Czech Republic under the name "Můj obchod" and in Poland under the name "Odido".More than 70 independent owners manage these stores and provide food and miscellaneous goods throughout the Slovak Republic. Behind this success is the fact that doing business in the alliance benefits suppliers, customers, and also consumers.

For our research was chosen the store "Môj obchod", located in the small village of Červeník on Hollého Street since 2014. The premises of this store are located in a relatively new apartment building on the ground floor, with a barrier-free entrance. The village Červeník has about 1700 inhabitants and there are two food stores situated relatively close to each other. "Môj obchod" store is mostly visited by the inhabitants of the village, which is dominated by regular customers who make their regular purchases here.

Table 1 Effect of Four Coffee Aromas on Emotional Response.

	Coffee House	Cappucino	Coffee and Cake	Coffe (pure)
Valence (polarity of emotions)	0.015	0.014	0.021	-0.01
Conscious evaluation	8.09	7.61	8.79	7.5

Note: Source: Results of the research.

Table 2 Wilcoxon Paired Test – Comparison of the Valence of Individual Aromas.

	Coffee House	Cappucino	Coffe and Cake	Coffee
Coffee	H_1	H_1	H_1	
Coffe and Cake	H_0	H_0		
Cappucino	H_0			
Cappucino				

Note: H0 – they are the same = there is no difference; H1 – they are different = there is a difference; Level of significance $\alpha = 0.01$. Source: Results of the research.

At the entrance to the store, there is a large cold counter with a selection of fresh meat products and cheeses, the store also offers bakery products, sweets, fruits, vegetables, various dairy products, soft drinks, drugstore, alcoholic beverages, and tobacco products. Customers can also buy draft wines or fresh desserts here. In this store, the assortment is different and according to the owner, tobacco products are sold the most. The owner strives to keep these foods attractive and encourages the people to shop mainly because of the proven quality. The store has been reconstructed several times since its inception and has undergone various changes.

Our research took place in the store "Môj obchod – Potraviny u Línajov".

Figure 1 shows an overview of the annual turnovers of the chosen "Môj obchod" store within the years 2016 to 2019. In 2016, the "Môj obchod" store was in operation for the third year with an annual turnover of 505,428 €. The store profits every year and its sales almost always grow significantly. In 2017, they rose to 571,222 €, but the owner had higher aims, so in 2018 introduced the sale of fresh meat to the store with a trained butcher. That year, sales rose rapidly to 635,165 €. Despite higher sales, this meat sale lasted only seven months and the owner decided to cancel these services from the store due to the high costs. The year 2019 showed lower sales due to the abolition of the butcher's shop, but the store continues to profit with a turnover of 609,987 €.

We researched in this store in two phases. The first phase without using the aroma diffuser; the second phase with its use. During both phases, a questionnaire survey was realized

We researched in the two phases in the store "Môj obchod – Potraviny u Línajov" The first phase without using the aroma diffuser; the second phase with its use. During both phases, a questionnaire survey was realized. The first phase – a questionnaire survey took place two weeks from March 1, 2020, to March 14, 2020. We placed 30 questionnaires in the store, which the salesmen offered to customers who had already made their purchase in this store. We contacted another 30 respondents again directly in the store after making the purchase and provided them with a tablet for filling in the form electronically using Google forms. In these weeks we managed to reach all 60 respondents. The questionnaire consisted of five classification questions (gender, age, monthly income, residence, and economic activity) and twelve factual questions, which were mostly related to the aroma of marketing and shopping feelings. In the questions, we asked the respondents how often they visit the store and how much time they spent in it, what influences them as much as possible when shopping and whether they are used to buying "TO GO" goods.

In the second phase – the questionnaire survey took place in a selected store using the aroma diffuser. We chose the "Cappuccino" aroma since it is most related to the types of coffee sold in the store and we assumed that it can evoke in the customers the feeling and wish to taste this product.

A survey with "Cappuccino" canned coffee – the aroma was held within the two weeks from March 15, 2020, to March 28, 2020. This phase of the survey was carried out in the same way as it was without the use of aroma with the help of shop assistants, who were given 30 pieces of printed questionnaires and by using the tablet, we obtained another 30 completed forms from the addressed respondents directly in the store. The questionnaire was identical to the one provided in the first two weeks. Together, we received the full number of responses from respondents in 60 forms.

Then we made several comparisons:

1) Comparison of sales volume and turnover of canned coffee. At this stage, we focused on whether there were changes before and during using the aroma in the store and we compared the individual periods by using a graphic representation and the table. We assume that the results we achieved in our research are largely influenced by the occurrence of Covid-19. People do not feel as comfortable in the store as under normal circumstances and also have to wear face masks, which reduce the intensity of the aroma.

2) Comparison of sales volume and turnover of selected confectionery. In selected periods before and during using the aroma in the store, we focused on observing changes in this segment of food, because the used aroma may also affect the sales of these products. We have shown these changes graphically and also in a table.

3) Monitoring the average values of purchases in the period when the aroma diffuser was not installed in the store and comparing them with the average values in the second stage of testing when the coffee aroma was used in the store. We focused on whether the application of the aroma caused changes in the final values of customer purchases. The receipts provided to us by our customers after their purchase also helped us in this evaluation.

The aroma diffuser "Aroma Streamer 650" was used to carry out the research. This device works with the principle of nebulization. A smooth and micro-fine nebulization of exclusive perfume compositions provides a quick aromatization of areas up to 100 m². It contains the integrated multi-function timer, which allowed us to set its operation every single day of testing.

"Cappuccino" aroma was sprayed into the air every four minutes for only 60 seconds. Of the three possible levels of setting the device, it was set to the second level.

The device was set from Monday to Saturday every day in the following time intervals – first mood: from 09:00 am to 11:00 am second mood: from 2:00 pm to 4:00 pm, third mood: from 5:00 pm to 7:00 pm.

On Sunday, we changed the device a bit because customers are in the store mostly from 9:30 am to 3:00 pm. The device was set as follows – first mood: from 9:00 am to 11:30 am second mood: from 12:30 pm to 2:00 pm, third mood: from 3:00 pm to 5:00 pm.

Used device: Aroma Streamer 650

Used software: MS Excell 2016

Statistical analysis

We used the following statistical methods to process the obtained data: Mann Whitney test, also called the Wilcoxon two-sample test – we compare the medians of two samples independent of each other. This test gives us the answer to the question of whether the difference between the medians (order of averages) of the two groups is statistically significant or only random. If the difference is significant (p <0.05), it means that there is a relationship between the sequence variable and the binary variable (group). The values of the characteristics can be calculated using the following relations (1, 2):

$$U_1 = m * n + \frac{m(m+1)}{2} - T_1 \quad (1)$$

$$U_2 = m * n + \frac{n(n+1)}{2} - T_2 \quad (2)$$

The relation (3) applies here:

$$U_1 + U_2 = m * n \quad (3)$$

which is used to control the calculation.

We reject the null hypothesis at the level of significance α in the case if $U_0 \leq U_\alpha$, where U_α are critical values of the Wilcoxon two-sample test.

If the possibility m >30 and n >20 occurs, then the test value will have the following form (4):

$$U = \frac{U_1 - \frac{1}{2}m*n}{\sqrt{\frac{m*n}{12}(m+n+1)}} \quad (4)$$

The alternative hypothesis is accepted if $|U| \geq u_\alpha$.

Friedman's test is a non-parametric alternative to repeated measures of ANOVA, where the assumption of normality is not acceptable. It is most often used in the case of an ordinally dependent variable. With this test, we verified whether the level of the specified character depends or does not depend on the change of conditions. It has the following form (5):

$$F = ((\frac{12}{nk(k+1)}\sum_{j=1}^{k} Rj2_{)-3n(k+1)} \quad (5)$$

Where:
n – number of respondents;
k – number of responses;
Rj – order of individual sample files.
H_0: The values have the same level and there are no statistically significant differences.
H_1 : The values do not have the same level and there are statistically significant differences.
We reject the hypothesis if F >x^2.

Nemenyi method – is based on the Kruskal-Wallis method on the one-way classification. The critical value is calculated by using the formula (6):

$$r_{\alpha,K,N\approx q\alpha,K\sqrt{\frac{K(K+1)}{6N}}} \quad (6)$$

Test of agreement of two differences (7,8)
H_0: $\pi 1=\pi 2$
H_1: $\pi 1\neq \pi 2$

π average – average proportion ; δ (π1 + π2) - selection error of the share difference ; has an N (0,1) distribution.

π average = (n1 * p1 + n2 * p2) / n1 + n2

$$\sigma_{(\pi 1+\pi 2)}=\sqrt{p*(1-p)*(\frac{1}{n1}*\frac{1}{n2})} \quad (7)$$

$$TCH=\frac{\pi 1-\pi 2}{\sigma} \quad (8)$$

KH = NORMSINV(1-ALFA)
TCH <KH : H_0 is accepted
TCH >KH : H_0 is rejected

RESULTS AND DISCUSSION

A survey of customer behavior before using the aroma diffuser in the chosen store

The survey involved 60 respondents, of which 35 were women and 25 men. According to the provided data, we can state that the selected store is mostly visited by women, up to 58% and 42% of visitors are men. After an interview with the employees of the store, we found out, that mainly women go to the store to buy the food for the entire family.

The differences between the individual age categories are not significant, i.e., all age categories over 18 years of age go to the store, approximately evenly. Total 16 respondents (27%) were from 26 to 33 years of age,14 respondents (23%) from 42 to 59 years and 13 respondents (20%) over 60 years old followed by 10 respondents (17%) aged from 34 to 41 years. The smallest group of respondents was created by 8people (13%) from18 to 25 years of age.

According to economic activity, the largest share of the respondents is employed -up to 22 people (37%). The second-largest group of respondents (13) were pensioners (22%). Furthermore, there are two groups with the same number of participants (6)representing a 10% share, namely students and mothers on maternity leave. The following smaller group consisted of 5 entrepreneurs (8%) Two groups with the same number of respondents, three working in the state sphere, and three unemployed with a 5% share. In the last place are 2 self-employed persons with a share of 3%.

Regarding the monthly income, the largest group of 26 respondents (44%), have a monthly income of up to 580 €. The second-largest group of 15 respondents (25%), had a monthly income from 581 € to 1,000 €. 11 respondents (18%) declared their monthly income between 1,001 € and 1,500 €. The last smallest group of 8 respondents (13%) had a monthly income of more than 1,501 €.

The chosen store is located on the ground floor of a block of flats in the countryside, which means that the majority of respondents (total 53, representing 88% share) come from the countryside. The other 7 respondents (12%) stated they live in the city and had to travel to visit the store. The majority of respondents visit the store several times a week (total 38, representing 63% share). As a result, people who get used to shopping in this store like to come back and make their regular smaller purchases here. Another group of customers visits the store several times a month (total of 15 respondents, representing 25% share). 6 respondents visit the store occasionally, which represents a 10% share. Only 1 person visits the store every day (1.7%)

When asked how much time they spend in the store, 36 respondents (60%) answered in the range of from 9 to 15 minutes. The second group consists of 21 respondents who spend less time in the store for up to 8 minutes. These respondents have a 35% share. The smallest group of respondents who spend more than 16 minutes in the store is 5% and consists of 3 people out of 60 respondents.

On average, customers stay in the store for a short time to buy and do not stay there arbitrarily. This may be because nothing in the store attracts them enough to spend there more time. The shop without any better experience for which they would stay in the store longer, which could be managed through implementing a certain level of marketing in the store.

Subsequently, the respondents had to determine the feelings of the atmosphere in the store on a five-point scale. The store was rated neutral by 20% of the surveyed customers and the largest percentage received a pleasant rating, up to 46.7%.

30% of respondents indicated the possibility of "very pleasant" feelings from the store, which may well affect our final research with the introduction of flavoring.

Anderson (2014) and **Vlahos (2007)** argue that the term "aroma marketing" can be used to set the mood, promote products or brand position **(Meng-Hsien, Cross and Childers, 2018)**.

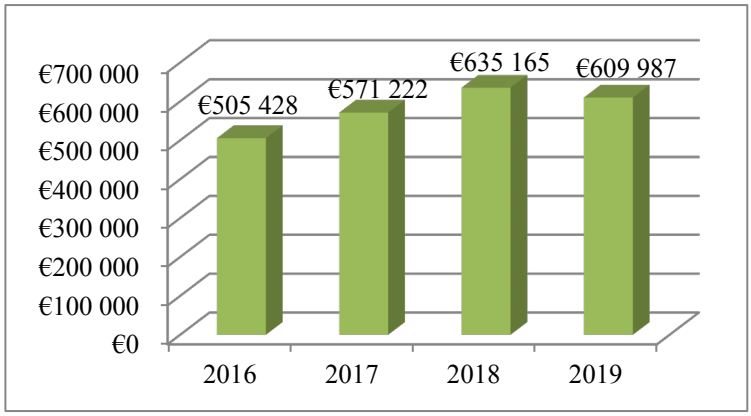

Figure 1 Overview of the Annual Turnover of the Chosen "Môj obchod" Store within the years 2016 to 2019 (in €). Note: Source: **Finstat, 2020**.

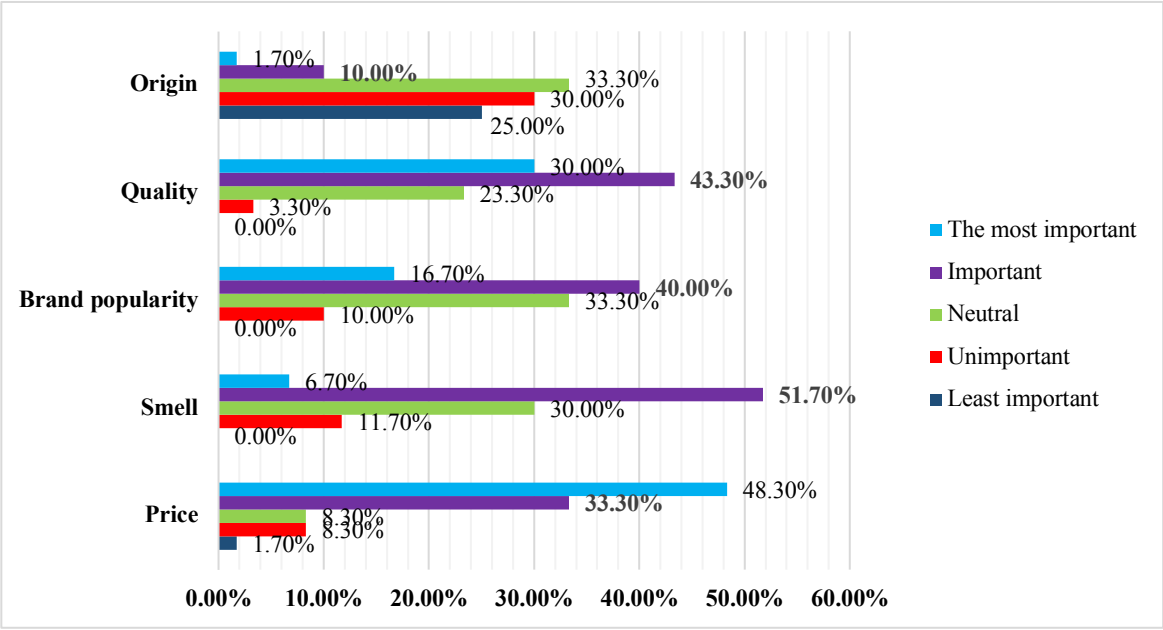

Figure 2 Factors Influencing the Purchase of Canned Coffee According to Importance. Note: Source: Results of the research.

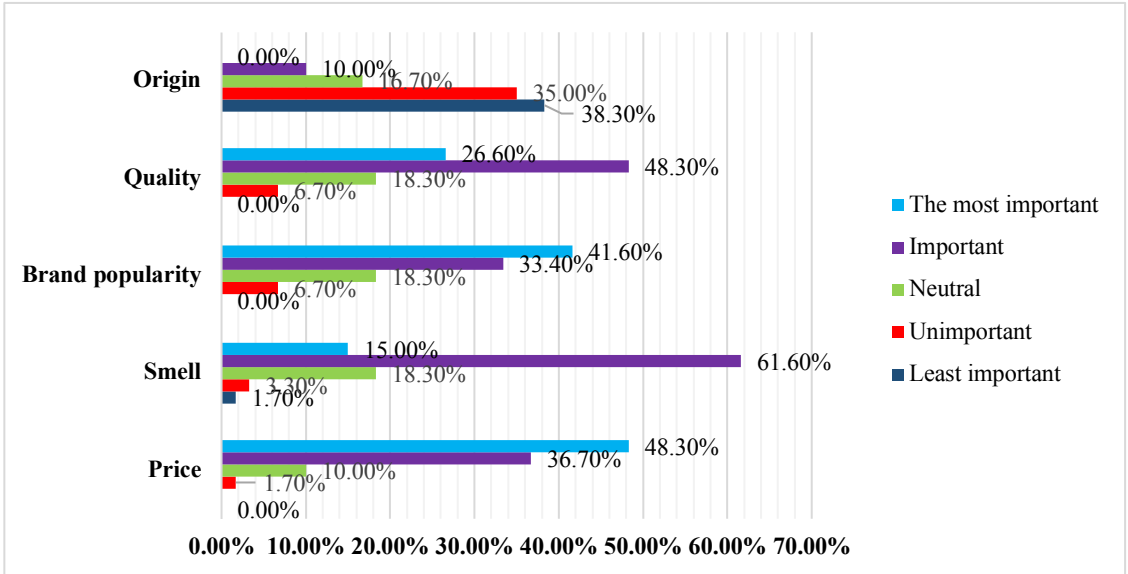

Figure 3 Factors Influencing the Purchase of Canned Coffee According to Importance after Using Aroma Diffuser. Note: Source: Results of the research.

For the research of the increase in turnover, it was subsequently necessary to identify what average price is the customer willing to pay at the local store. As it is a smaller store, people usually make smaller purchases for households, and the obtained values are also related to this fact. 22 respondents (36.7%) buy their goods in the amount of 11 € to 20 €. The second most common group of purchases is up to 10 €, as stated by 20 respondents (33.3%). Furthermore, the purchase from 21 € to 50 € proved 18 respondents (30%). Customers state that their regular purchases in this store are usually not above the level of 50 €. We can assume that customers are willing to spend more when shopping in larger supermarkets, where they have a wider choice of goods in larger quantities and go to the selected store earlier for the goods they consume daily and replenish it.

Even before using the aroma diffuser, there is a certain smell in the store, e.g. the smell of fresh-baked pastries.

To choose the correct aroma is not always as easy as it may seem at first glance. If we want to support sales of confectionery, it is not enough to add a chocolate aroma **(Vysekalová, 2014)**.

According to **Gobe (2010)**, the smell of a product creates a characteristic feature and can be easily identified by the customer. If the product is "pleasant-smelling", it looks positive and provides a space for a favorable identity. Every single smell plays a different role. For example, peppermint and lemon can boost alertness and energy; cedar and lavender can reduce tension.

Thus, the largest group of respondents (total 32, representing 53.3%) stated that they felt a "pleasant smell" at the entrance. 21 respondents (35%) stated that they consider the smell to be "very pleasant". Another 7 (11.7%) respondents felt a "neutral smell" at the grade. We can state that customers chose the most "pleasant" smell most often due to the natural scents that were carried by the store.

We also asked about other aromas to which the seller should appeal the most, which is sales support for both sales and sales staff **(Přikrylová and Jahodová, 2010)**.

Respondents could mark several answers to a given question. Based on the obtained data, the seller should pay the most attention to the visual side of the store and goods. The sense of sight was marked by up to 43 respondents (71.7%), the second sense that should be pointed out was the smell, which was marked 39 times (65%). Taste and hearing took 20% share and customers pay the least attention to the tactile sense (only 3.3%). The visual side of the store is the most important when the customer moves around the store and searches for the selected goods. If we add the aroma to this and combine these two investigated quantities, it is more likely that the customer will buy the goods.

Subsequently, we focused on "TO GO" goods. Respondents buy "TO GO" goods to a large extent, most of them chose to buy them "a few times a week", up to 32 respondents (53%). The possibility "sometimes" was indicated by 25 respondents (42%) and 3 respondents (5%) buy "TO GO" goods every day. We assume that these goods are bought by people mainly due to the hectic time in which we live. For example, if they buy canned coffee, they save time for making coffee at home. Some people may have become accustomed to this way of life, so they are buying such goods more and more often. It's also a good way to enjoy shopping by choosing such a small, not very expensive item.

As all 60 respondents stated that they buy all these goods. The largest number of respondents 37 (61.7%) stated that they buy sweets. The second most purchased is canned coffee, which was marked by 29 respondents (48.30%). Furthermore, the chips were marked by 24 respondents (40%), sandwiches by 14 respondents (23.3%) and the least purchased are energy drinks with 10 marks (16.7%).

Then we focused only on the canned coffee and which factors influence customers when buying it. Respondents could assign degrees of importance to individual factors. All factors available to them for selection are listed in Figure 2.

The most important factor that would affect them when buying was the price, which was indicated by up to 48.3% of respondents. They consider quality to be the second most important factor with 30% a share. This is followed by the popularity of the brand 16.7%, then the smell of 6.7%, and the origin was indicated by only 1.7% of respondents. In the least important area, there was an origin with a significant value of 25%. From the above data, we can say that people are most affected by price, product quality, brand popularity, and smell. The least important factor for customers has become the origin of the product.

We then focused only on canned coffee and which factors influence customers when buying it. Respondents could assign degrees of importance to individual factors. All factors available to them for selection are listed in Figure 2. The most important factor that would affect them when buying was the price, which was indicated by up to 48.3% of respondents. They consider quality to be the second most important factor with a 30% share. This is followed by the popularity of the brand 16.7%, then the smell 6.7%, and the origin was indicated by only 1.7% of respondents. In the least important area, there was an origin with a significant value of 25%. From the above data can be concluded that people are most affected by price, product quality, brand popularity, and smell. The least important factor for customers has become the origin of the product.

For verification, we also used research assumption 1, where we assumed that before using the aroma diffuser, the customers will perceive individual factors differently. To verify this assumption, we used the Friedman test with Nemenyi pairwise comparison method. We verified the assumption of a selected sample of respondents. The value of the test characteristic (0.05) is higher than the critical value (0.001), therefore we can state that the customers rated the individual factors differently before using the aroma diffuser. However, up to 78% of customers said they did not buy canned coffee.

A survey of customer behavior after using the aroma diffuser in the chosen store

At this stage of the survey, the chosen store is visited mostly by the customers aged 42 to 59 (total 19 respondents, 32%), followed by the second group of 15 respondents (25%) from 26 to 33 years. Furthermore, there are 12 respondents (20%) over the age of 60 and 9 respondents (15%) from a group of 34 to 41 years old. The smallest group of 5 respondents (8%) are people aged from 18 to 25 years. There are only small differences in this study compared to the first stage of the survey.

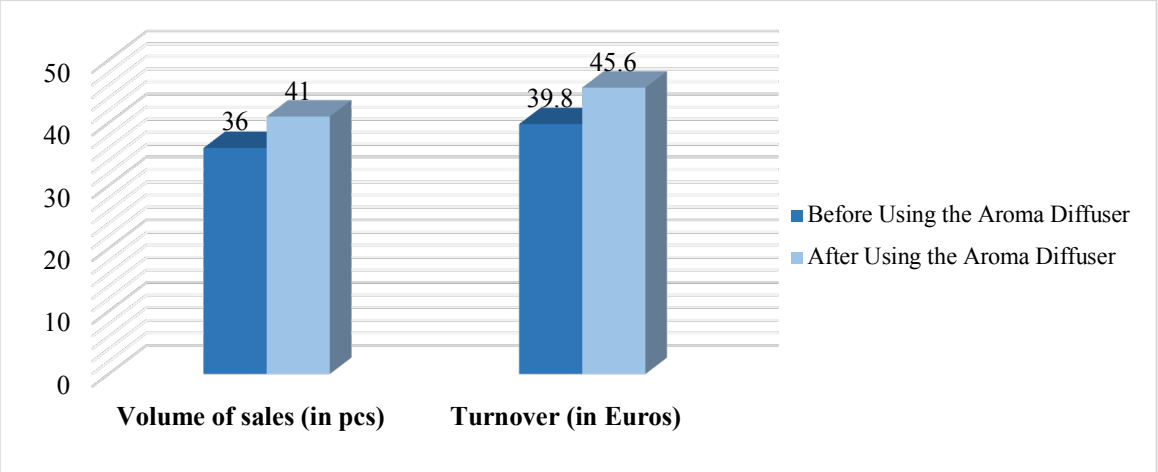

Figure 4 Volume of Sales and Turnover of Canned Coffee Before and After Using the Aroma Diffuser (in €). Note: Source: Results of the research.

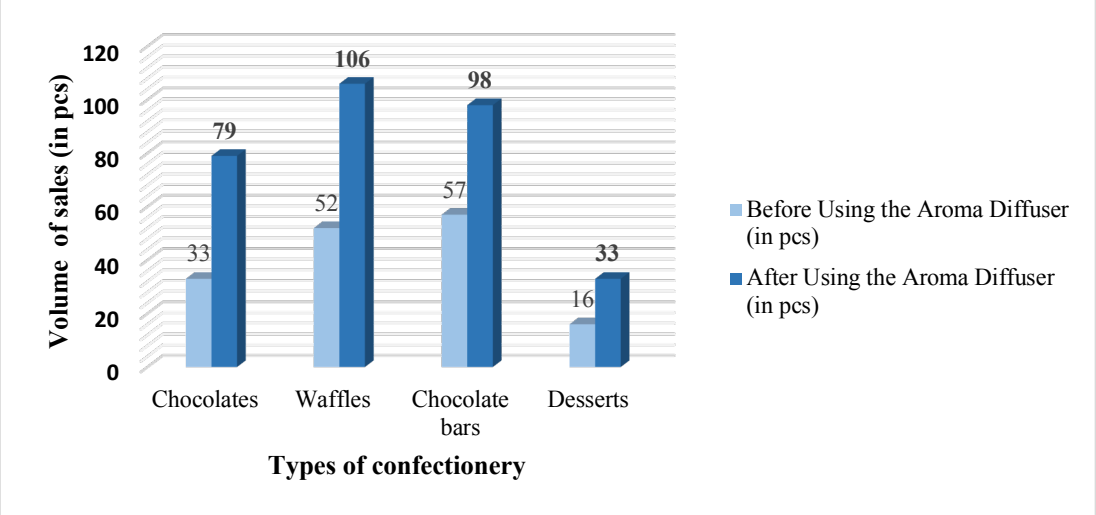

Figure 5 Volume of Sales of Confectionery Before and After Using the Aroma Diffuser (in pcs). Note: Source: Results of the research.

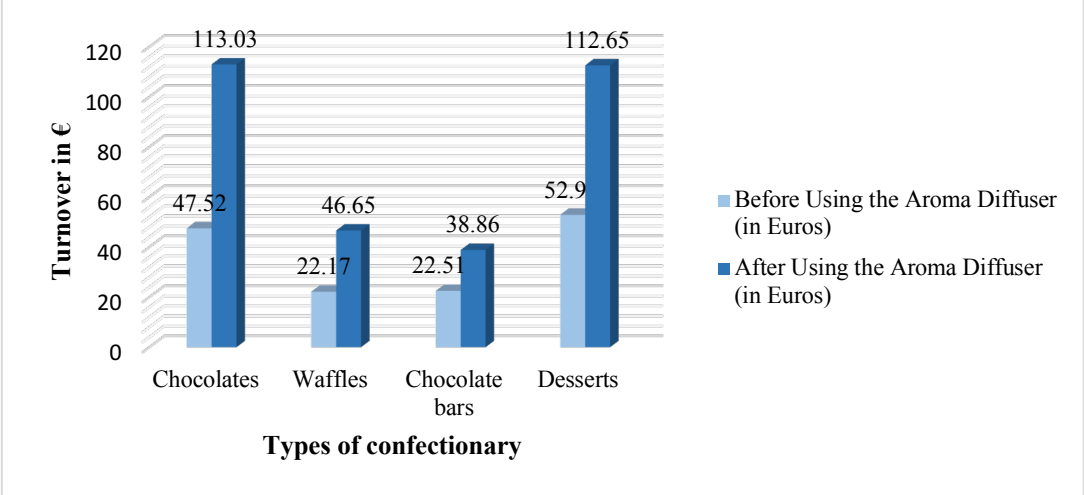

Figure 6 Turnover of Confectionery Before and After Using the Aroma Diffuser (in €). Note: Source: Results of the research.

As many as 26 respondents (43%) were from the group of employed people, which is almost half of the respondents, and compared to the previous stage of the survey, this number has increased by 6%. The second-largest group with 13 (22%) are pensioners. Groups ranging from 8% to 5% are students, mothers on maternity leave, entrepreneurs, self-employed and unemployed people.

A total of 23 respondents (38%) stated that their monthly income ranges from 581 € to 1,000 €, the increase is 13%, compared to the first stage of the survey. 22 respondents (37%) stated their monthly income is up to 580 €. Furthermore, 11 respondents (18%) reported their income is from 1,001 € to 1,500 € and the remaining 4 respondents (7%) have an income higher than 1,501 € per month.

Most customers visit the store several times a week (total 38, representing 63%). 13 customers (22%) visit the store several times a month, which is 7% more than in the first stage of the survey. Daily visit to the store was marked by 5 respondents (8%), this number also increased by 6%. Only 4 surveyed customers (7%) visit the store occasionally.

Regarding the time spent in the store, more than 38 respondents (63%) said they would stay in the store from 9 to 15 minutes. A total of 12 respondents (20%) stated that they spend more than 16 minutes on their purchase, which is 15% more than in the first stage of the survey. Due to the situation with the Covid-19 virus, people decided to visit the store with the intention of larger one-time purchases, which last significantly longer than usual. The last group consists of customers who visit the store in the interval from 1 to 8 minutes and there were 10 of them (17%).

Fragrances or aromas thus support the customer's stay in the store **(Madzharov, Block and Morrin, 2015)**.

The change in the evaluation of the feelings from the atmosphere of the store occurred with the possibility of "unpleasant", which was marked by 2 respondents (3%). We believe that due to the smell of cleaning and disinfecting detergents, which were used more in the store on the given days. A total of 7 respondents (12%) had "neutral" feelings about the atmosphere. The second largest group consisted of the surveyed customers who marked a "pleasant atmosphere" (Total 23, representing 38%). The most marked was "very pleasant" atmosphere in the store, by up to 28 respondents (47%), which is 14% more than before using the aroma diffuser in the store. We can assume that the used aroma had a positive effect on the senses of customers.

We also verified this factor by using Mann Whitney – a one-sided test, based on Assumption 2. We assumed that the feelings of the store atmosphere before and after using the aroma diffuser are different. The critical value was higher (0.09) than the test characteristic (0.05), which means there is no difference between the evaluation of the customer feelings before and after using the aroma diffuser in the store.

As many as 31 (52%) customers stated that they usually spend between 11 € and 20 € on their purchases, there is an increase compared to the first stage of the survey, by up to 15%. On the contrary, purchases up to 10 € fell by 2% and this answer was marked by 17 respondents (28%). Average purchase from 21 € to 50 € was marked by 11 respondents (18%) and more than 51€ spent just 1 respondent (2%). Because the Covid-19 pandemic broke out much more intensively in the second stage of testing, we can assume that people were responsible and went to the store only for things that they needed for everyday life. This could mean that the number of their purchases could rise to 20 €, which can hold food without which households cannot function normally.

The smell in the store was marked as "unpleasant" by up to 4 respondents (7%) and we can assume that it was again caused by disinfectants and cleaning detergents, which were used excessively these days. A total of 3 respondents (5%) consider the "neutral" smell in the store. The "pleasant" smell in the store was for 18 respondents (30%) and the highest share was achieved by a "very pleasant" smell, which was marked by up to 35 respondents (58%), which represents an increase of 23%. It very much depended on when the questionnaire was filled in and when the aroma was activated in the store. In the evening, the store was cleaned more intensively.

In assumption 3 was assumed there is a difference between the impression of smell in the store before and after using the aroma diffuser.

We have used again the Mann Whitney – a one-sided test. The value of the test characteristic is higher (0.05) than the critical value (0.02), therefore can be stated, that customers felt the difference after applying the aroma to the store. According to the obtained data, the seller should pay attention to the sight and visual side of the store, as up to 48 respondents (80%) marked this answer, which is 37% more than in the first stage of the survey. The second one is the smell with 31 answers (52%), marked by 13% fewer people. The taste was marked by 16 respondents (27%) and hearing by 5 respondents (8%).

The largest share among the answers which smell in the store attracted the customer the most, was the smell of fresh-baked pastries", which was marked by up to 31 respondents (51%), this group increased by 9%. The second-largest group was created by the answers that the respondents could complete themselves. This group of responses has undergone the most significant change. The most common response is the "smell of chocolate", which occurs up to 16 times (27%). Furthermore, it is "disinfectant or cleaning products", which was mentioned by 5 respondents (8%) and 1 respondent (2%) stated the smell of "cappuccino".

Aroma has a remarkable ability to make customers look around the store longer, spend more money, and return to the store more often. Emotions and memory are affected by the power of scents in very close ties. Find the right scent and you can get around rational thoughts. In-store testing shows that aroma can be the holy grail. It has the power to encourage the customer to join a new brand and remain loyal **(Calvo-Porral, Ruiz-Vega, and Lévy-Mangin, 2018)**.

The most marked answer when buying "TO GO" goods were that the respondents buy these goods a few times a week (total of 38 respondents, representing 64%), compared to the first stage there is an increase of 11%.

14 respondents (23%) indicated that they buy these goods only sometimes, which caused a decrease of 19%. "TO GO" goods are bought daily by 8 respondents (13%), compared to the first stage there is an increase of 8%.

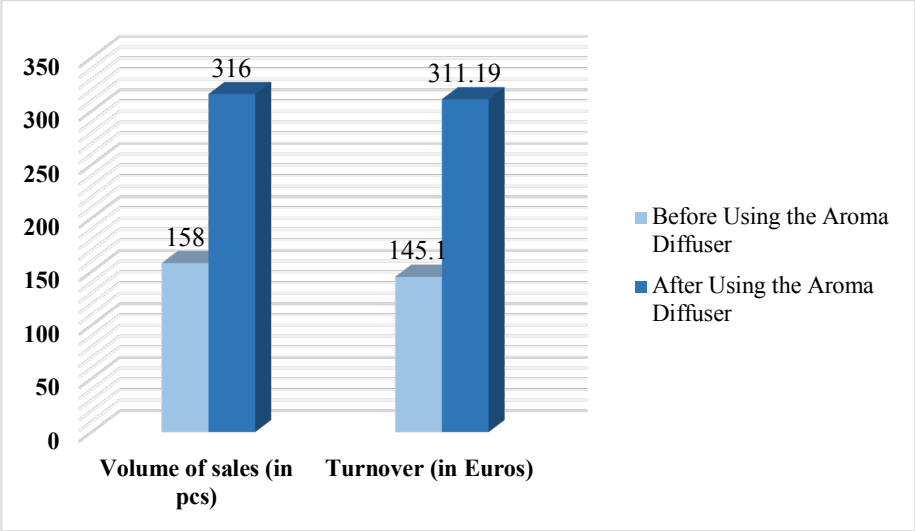

Figure 7 The Total volume of Sales and Turnover from Confectionery Before and After Using the Aroma Diffuser. Note: Source: Results of the research.

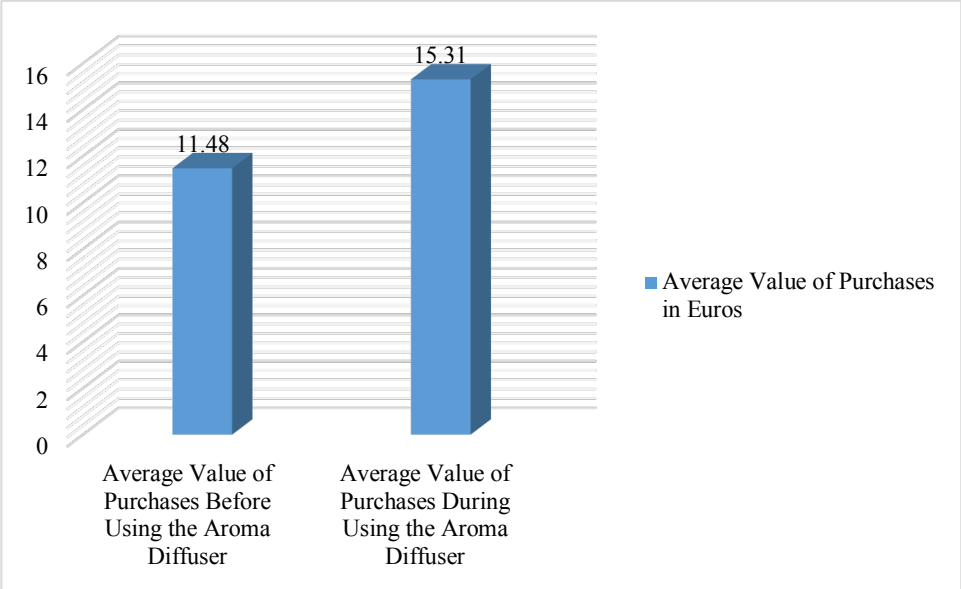

Figure 8 Average Value of Purchase Before and After Using the Aroma Diffuser in €. Note: Source: Results of the research.

Table 3 Types of Selected Canned Coffees – Their Quantity (in pcs) and Turnover in €.

	Quantity in pieces		Difference	Turnover in Euros		Difference
	Before	After		Before	After	
Nescafé	12	14	**2**	15	17.5	**2.5**
Energy coffee	8	11	**3**	8.8	12.1	**3.3**
Ice coffee Landessa	16	16	**0**	16	16	**0**

Note: Source: Results of the research.

As many as 45 respondents (75%) said they prefer to buy sweets. This percentage increased by 38% compared to the first stage of testing, which is more than half. We can assume that they were influenced by the sweet aroma they felt when shopping. Canned coffee and chips have a relatively equal proportion of 25 (42%) and 26 (43%) respondents. The sandwiches and baguettes were mentioned by 13 respondents (22%), and the least marked item was energy drinks, which were mentioned by 6 respondents (10%).

As in the first stage of the survey, respondents had the opportunity to assess the importance of the factors that would make them buy canned coffee in the selected store. That is the factor that would motivate them the most to buy. Respondents had the opportunity to assign degrees of importance to individual factors. Most respondents stated that they consider the price to be the most important factor, up to 48.3%, and the second one is the brand popularity. The smell was marked as important by up to 61.6%, which represents a 10% increase. People consider the origin of the product to be the least important (38.3%) and the insignificant factor (35%), compared to the first survey, these percentages increased by 13% and 5%. With a value of 18.3%, the respondents described the quality, the brand popularity, and the smell as neutral. We can state that customers consider the price, brand popularity, quality, and smell to be a really important factor. These results are shown in Figure 3.

Assumption 4: We assume that the significance of the smell after using the aroma diffuser is more important.

We have used the Mann Whitney again – a one-sided test. Where we got a critical value of 0.01, which is less than the test characteristic of 0.05, which proved that customers found the smell more important after using the aroma diffuser in the store.

After using the aroma diffuser in the store, we find that sales of canned coffee increased slightly. 19 respondents (32%) stated that canned coffee is in their shopping cart and this number has increased by 10%. A total of 41 respondents (68%) stated that they did not buy canned coffee, which is 10% less than before applying the aroma.

The total volume of canned coffee sales and turnover before and after using the aroma diffuser is shown in Figure 4. The sales volume before applying the aroma was 36 pieces, while after applying the aroma it was 41 cans, which means a small increase in sales and also in the comparison of turnover. Sales increased by 5 pieces together from all selected types of coffee. Before using the aroma diffuser, the turnover was 39.8 € and after using the aroma diffuser, the turnover of these goods was 45.6 €, which is an increase of 5.8 €.

We can assume that used aroma had a positive effect on sales, but not very significant. Finally, the customers combined the aroma we used with sweets. In Table 3, we described the specific types of canned coffee that were observed, namely Nescafé coffee, Energy coffee, and Ice coffee Landessa.

"TO GO" goods also include confectionery, which is why we deal with them in the submitted paper.

In the chosen two periods, we observed the volume of sales and turnover of a certain segment of goods concerning the placement of the aroma diffuser directly in the store. Based on the recording of individual items, we were able to observe changes in the volume of sales and turnover, to find out where was the largest increase in sold items.

Figure 5 shows the volume of sales of the chosen confectionery before and after using the aroma diffuser. All available and obtained data show us that the sales volume of goods on which we focused, increased during the period of applying the aroma in the store. Of all examined items, the sales of waffles increased the most, by 54 pieces, while the original sale was 52 pieces. Another big increase was the chocolates from the original 33 sold pieces to 79 pieces. Chocolate bars increased from 57 pieces to 98 pieces. Desserts also increased the volume of sales from 16 to 33 desserts. Based on the data obtained in practice, we can state that for selected types of confectionery, the aroma has a positive effect on the shopping behavior of consumers and thus helps to increase the volume of purchases

Figure 5 shows the volume of sales of the chosen confectionery before and after using the aroma diffuser. All available and obtained data show us that the sales volume of goods on which we focused, increased during the period of applying the aroma in the store. Of all examined items, the sales of waffles increased the most, by 54 pieces, while the original sale was 52 pieces. Another big increase was the chocolates from the original 33 sold pieces to 79 pieces. Chocolate bars increased from 57 pieces to 98 pieces. Desserts also increased the volume of sales from 16 to 33 desserts. Based on the data obtained in practice, we can state that for selected types of confectionery, the aroma has a positive effect on the shopping behavior of consumers and thus helps to increase the volume of purchases.

Due to the increase in the volume of sales, the turnover from confectionery also increased. This is clearly shown in Figure 6. Turnover from sold chocolate increased the most, by up to 65.51 € and this difference is higher than the turnover from chocolate before applying aroma. The second-largest increase was in desserts, where the store earned 59.75 € more than in the first stage of our survey, followed by the waffles which turnover increased from 22.17 € to 46.65 €. The lowest increase was noticed in chocolate bars, in which turnover increased by 16.35 €.

Table 4 lists all the items on which we examined the volume of their sales and turnover. During applying the aroma, Mila waffles were sold the most, where the difference was 26 pieces. In the chocolate segment, we recorded a large increase of up to 17 pieces of Milka milk chocolate. Regarding the turnover, we recorded the most significant difference by 25 € in Milk Metro Premium chocolate. Another important item was the Tatiana chocolate candies box with an increase of 19.25 €. In short, we can say that our research has yielded positive results and the use of such marketing tools is beneficial for the store.

Figure 7 shows the total volume of sales and turnover from confectionery before and after using the aroma diffuser. Without the used aroma 158 pieces of selected confectionery were sold, and during the use of the aroma, the volume of sales increased to 316 pieces. Before aromatization was turnover of confectionery 145.1 € while applying the aroma was recorded an increase of turnover to 311.19 €. Through this research, we concluded that the aroma of the store has a positive effect on the volume of sales and turnover of confectionery in this store. The volume of sales of confectionery increased by exactly half, by 158 pieces, and the turnover increased by 166.09 €.

Table 4 Types of Selected Confectionery– Their Quantity (in pcs) and Turnover (in €).

	Quantity in pieces			Turnover in Euros		
	Before	**After**	**Difference**	**Before**	**After**	**Difference**
Types of Selected Confectionery			**Chocolates**			
Milk Metro Premium (80 g)	6	16	**10**	15	40	**25**
Figaro milk chocolate (100 g)	10	24	**14**	9.9	23.76	**13.86**
Milka milk chocolate (100 g)	9	26	**17**	9.9	28.6	**18.7**
Študentská pečať milk chocolate (180 g)	8	13	**5**	12.72	20.67	**7.95**
			Waffles			
Types of Selected Confectionery	**Before**	**After**	**Difference**	**Before**	**After**	**Difference**
Mila (50 g)	12	38	**26**	6	19	**13**
Kakaové rezy (50 g)	14	26	**12**	5.88	10.92	**5.04**
Kávenky (50 g)	17	29	**12**	7.14	12.18	**5.04**
Princezky (80 g)	9	13	**4**	3.15	4.55	**1.4**
			Chocolate Bars			
Types of Selected Confectionery	**Before**	**After**	**Difference**	**Before**	**After**	**Difference**
Kofila (35 g)	15	28	**13**	7.05	13.16	**6.11**
Yami (25 g)	20	32	**12**	5.6	8.96	**3.36**
Orion KOKO (35 g)	14	26	**12**	5.46	10.14	**4.68**
Kaštany (45 g)	8	12	**4**	4.4	6.6	**2.2**
			Desserts			
Types of Selected Confectionery	**Before**	**After**	**Difference**	**Before**	**After**	**Difference**
Tatiana (172 g)	6	11	**5**	23.1	42.35	**19.25**
Modré z neba (150 g)	2	6	**4**	9.7	29.1	**19.4**
Orion orieškový sen (87 g)	7	12	**5**	17.15	29.4	**12.25**
Pergale Milk chocolate (187 g)	1	4	**3**	2.95	11.8	**8.85**

Note: Source: Results of the research.

During the survey, we also found out the average values of purchases from customers. In Figure 8 we can see the average value of the purchase before and after using the aroma diffuser. Before applying the aroma, the average value of the purchase was 11.48 €. After applying the aroma in the second stage of the survey, this average purchase value increased by 3.83 € to 15.31 €. After comparing the found values, we can state that the aroma led customers to buy the items that attracted them due to their sensory perception, and the value of the average purchase increased slightly. We can state that aroma is one of the factors that have a positive effect on shopping behavior.

Based on the results of the research, we can state that using the aroma diffuser in the store brought a clear benefit in increasing sales of either canned coffee or confectionery. However, it is necessary to handle the aroma diffuser unit wisely, because when multiple aromas are mixed, it can also bring the opposite effect. To place the aroma diffuser in the store could also be beneficial for such products that cannot produce their natural scent, for example, due to packaging, and thus increase their attractiveness to the customer. Another reason for applying the aroma is to focus on products that are selling less and thus support their sale.

Merchants are increasingly using the surrounding aroma as a strategic tool to differentiate themselves from the competition, attract customers, stimulate sales, influence moods and create an overall pleasant and unforgettable shopping experience **(Madzharov, Block and Morrin, 2015)**.

The use of aromas is a new generation of communication tool that measures the impact on the consumer and the impact that is created on the customer and interactivity in the store **(Labská, 2012; Rimkute, Moraes and Ferreira, 2016)**.

Aroma marketing, or so-called Scent marketing, can be used in two areas. The first is ambient scenting, this term means filling the space with a suitable type of scent and the other area is scent branding, which can be used to create a specific scent. It identifies a brand, product, institution, company, or environment. The scent should be selected and

deployed to match and perfectly adapt to the environment and context **(Naščáková and Danková, 2017; Minsky, Fahey and Fabrigas, 2018)**.

There also exist the products that represent a characteristic feature of a particular product and the main reason for buying such a product is mainly the smell **(Krishna, 2013)**.

It is important to search for and use new forms of marketing, as we are increasingly oversaturated with advertising and this has made the customers more immune to traditional marketing **(Berčík, 2017)**.

CONCLUSION

The environment in which the customer is located during his purchase has an intensive effect on him and also affects sales. One of the modern tools used in commercial operations to make the products visible and also to support their sales is aroma marketing. This type of marketing deals with how aromas can stimulate human senses and emotions and purposefully change the customer's behavior and improve the shopping experience.

The important thing is the representation of the senses when shopping, and the human brain often does not realize that the smell released in the store can keep the customer in the store for longer, or to cause the desire to buy the product that was not originally planned to buy.

To examine consumer behavior a questionnaire survey was conducted directly in the store where the aroma diffuser was used. Thanks to this survey, we wanted to find out to what extent customers will be affected by the aroma released into the store and whether it will affect their shopping behavior. There were no significant differences in the number of store visits and the time spent in the store during the purchase before and after using the aroma diffuser. The only change was that a few customers spent more than 16 minutes in the store applying the aroma, which was usually up to 15 minutes before using the aroma diffuser in the store. Customers stated that during applying the aroma, in addition to the staff, they were also more affected by the smell in the store. When evaluating the feelings from the atmosphere of the store before and after using the aroma diffuser, we assumed that the feelings will be evaluated differently. In addition to the questionnaire survey, we also used the Mann Whitney test. However, this test showed us that the customers did not notice the difference between the periods and perceive the atmosphere in the same way. However, we noticed that two respondents described the feelings about the store as unpleasant and we believe that this is due to excessive disinfection of the store in the evening due to the occurrence of Covid-19 and strict hygiene regulations of the Slovak government. This fact is followed by the fact that the smell in the store was described by 4 respondents as unpleasant and we think that it is also due to disinfectants. Nevertheless, customers perceive the smell in the store better after using the aroma diffuser, and this was also confirmed by the statistical Mann Whitney test. According to the obtained results, customers believe that the seller should appeal to sight, followed by smell and taste when organizing the store.

When choosing the aroma, we tried to make it as similar as possible to the aroma of cappuccino. Nevertheless, during the second stage of the survey, customers in the store smelled the most the fresh-baked pastries also the smell of chocolate. This refuted our assumption of an increase in coffee sales. We were also interested in whether customers buy "TO GO" goods. The largest group of respondents buys such goods a few times a week, mostly sweets, canned coffee, and chips. We were interested in which factor customers consider most important when buying the canned coffee. By statistical testing using the Friedman test with the Nemmenyi method, we confirmed the fact that after using the aroma diffuser in the store, the respondents consider the smell as important and significant. The survey showed us that they consider the origin of the product to be the least important factor. By using the pairwise comparison test we found that using the aroma diffuser did not affect the sales of canned coffee. This refuted our assumption that the aroma we applied to the air would mainly affect the sales of canned coffee.

The highest increase in the volume of sales of confectionery during aromatization was shown in waffles, chocolates, chocolate bars, and desserts. From the obtained data, we can state that the aroma was one of the factors that strengthened sales and thus contributed favorably to the increase in sales of these food products which more than doubled. We dare to say that aroma as a marketing tool appears to be a positive tool and can bring economic prosperity in the future. We also focused our attention on whether the average value of purchases by customers has increased. According to the respondents, we concluded that the average value of the purchase increased from the original 11.48 € to 15.31 €, which may also be caused by the fact, that customers added to the cart, for example, sweets, because they were affected by used aroma in the store. We can state that customers increased the value of their purchases also due to the situation with the Covid-19 virus and increased their food stocks in households. Through aroma marketing, it is possible to build a positive relationship of an individual to a brand, product, or service. It is likely that based on the smell, the customers will keep this relationship in mind and will return to the purchase.

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CHEMICAL PROPERTIES AND ACCEPTANCE IN THE BISCUIT FORMULA OF BELITUNG TARO (*XANTHOSOMA SAGITTIFOLIUM)* WITH ADDITION OF ANT NEST TUBERS (*HYDNOPHYTUM FORMICARUM)* PLANT

Anjar Briliannita, La Supu

ABSTRACT
This study was aimed to analyze the nutrients content, antioxidant activity, and acceptance of the biscuit formula of Belitung taro (X*anthosoma sagittifolium*) with various parts of ant nest tubers (*Hydnophytum formicarum*) plant. Antioxidant activity was found by the DPPH method, nutrient content by the AOAC method, and sensory evaluation analysis by 30 untrained panelists. The results of this study found out that each biscuit formula (35.25 grams/73.60% antioxidant activity) contained 130 kcal of energy, carbohydrates of 59.08 ±0.16%, the protein of 4.13 ±0.06%, fat of 22.17 ±0.19% so that it was able to compensate for 5.97 – 6.57% of total energy requirements based on the Nutrition Adequacy Rate for the Elderly per day. The overall preference level in the biscuit formula by untrained panelists was 4.10 ±0.72 (rather like-like). The addition of 7.5% ant nest tuber powder in the biscuit formula had an antioxidant activity (DPPH) of 73.60 ±0.36%. The most preferred biscuit formula was the C013 biscuit formula (addition of 6% ant nest tuber powder per gram biscuits), with the higher antioxidant activity, and its nutrient content to compensate for the nutrition adequacy rate for the elderly per day.

Keywords: biscuits; Belitung taro; ant nests tuber; antioxidant activity; organoleptic test

INTRODUCTION
Belitung taro or kimpul *(Xanthosoma sagittifolium*) is a tropical plant derived from *Araceae* family, containing high starch ranging between 22% to 40%, and because of that, Belitung taro or kimpul is a good source of carbohydrate **(Pérez, Schultz and de Delahaye, 2005**). Small starch granules from Belitung or taro *(Xanthosoma sagittifolium*), from the aspect of digestibility, starch with a small granule size was easier to digest. Other significant nutritional content in Belitung or kimpul taro *(Xanthosoma sagittifolium*) was protein one level higher than sweet potato and cassava (every 100 grams of taro or Belitung *(Xanthosoma sagittifolium*) contains 1.9 g of protein while cassava contains 1.2 g and sweet potato 1.8 g. Belitung taro tubers contain 0.32 g total phenolics per 100 grams of material; 0.26 g flavonoids per 100 grams of material and activity *scavenging* better using DPPH testing (78.22 ±0.56%), *hydroxyl radical* (69.11 ±0.21%), superoxide radicals (83.27 ±0.08%), and ABTS *radical cations* (76.11±0.07%) **(Nishanthini and Mohan, 2012)**.

Meanwhile, *Hydnophytum formicarum* or ant nests tuber plant from West Papua were used in research as a source of antioxidants. This plant was hereditarily consumed in the form of boiled tuber powder and was used to cure diseases such as cancer, tumors, gout, migraine, periodontitis, coronary heart disease, tuberculosis, and leukemia, but it was also used to treat diabetes mellitus. Significantly, the ethyl acetate extract *Hydnophytum formicarum* was also the most potent antioxidant, showing 83.31% of radical scavenging activity with IC_{50} 8.40 $\mu g.mL^{-1}$ in the DPPH assay. The other extracts display weak to moderate antioxidative activities, ranging from 28.60 – 56.80% radical scavenging. The SOD assay shows that methanol extract exhibits the highest activity (74.19% inhibition of superoxide radical). The dichloromethane and ethyl acetate extracts display comparable SOD activity. The promising bioactivities of the crude ethyl acetate extract guided the first isolation of bioactive flavonoid and phenolic compounds: isoliquiritigenin, protocatechualdehyde, butin, and butein from this species, we propose that *Hydnophytum formicarum* Jack. can serve as a new source enriched with potent antioxidative and antimicrobial agents **(Prachayasittikul et al., 2008)**.

Based on another study *H. formicarum* tuber from Setiu wetland and Muara Rupit showed very good potency as antibacterial and antioxidant agents**.** All samples showed high DPPH free radical scavenging activity (IC50 less than 10 $\mu g.mL^{-1}$). Via MTS assay, no cytotoxic activity of all samples was observed against HeLa cells. Only a fraction from Setiu Wetland showed very strong cytotoxic activity against MCF-7 cells (IC50 = 2 $\mu g.mL^{-1}$) and its morphological features stained by Annexin-V/PI and DAPI

a

b c

Figure 1 *Hydnophytum formicarum* and ant nest tuber powder
Note: a) ant nest tuber plant (*Hydnophytum formicarum*) raw; b) ant nest tuber plant (*Hydnophytum formicarum*) dry; c) ant nest tuber powder plant (*Hydnophytum formicarum*).

showed that the cell death was mediated by apoptosis. **(Andriani et al., 2017)**.

Studies were conducted on the structure and rheological properties of three cocoyam varieties *Xanthosoma sagittifolium* (red-flesh), *Xanthosonia sagittifolium* (white-flesh) and *Colocasia esculenta* starches and raphides in an attempt to characterize them. No distinct variation was observed in starch granule sizes of the two *Xanthosoma* species. Starch granules sizes in the ranges of 0.74 – 1.19 and 0.74 – 1.10 μm were obtained for the *Xanthosoma* species (red tubers) and *Xanthosoma* species (white tubers), respectively. Significantly smaller sizes (0.05 – 0.08 μm) of starch granules were observed for *Colocasia esculenta*. Peak viscosity was highest in the *X. sagittifolium* (red-flesh) variety while the white-flesh variety showed the least tendency to retrogradation **(Sefa-Dedeh and Kofi-Agyir Sackey, 2002)**. Based its study *X. sagittifolium* species white tubers have a low glycemic index because of their high amylose starches. The glycemic index has been categorized by Miller as low GI is pounds

a b

Figure 2 Beiltung taro and flour.
Note: a) Belitung taro *xanthosoma sagittifolium* raw; b) Belitung taro *xanthosoma sagittifolium* flour.

Figure 3 Biscuits made with addition of Beiltung taro flour and ant nest tuber flour.

55, moderate GI is between 56 – 69 and high GI is ≥70. Glycemic index (GI) has been widely used in the management of blood sugar levels among diabetes, however; in the South Pacific, very little information regarding the GI of local foods is made available. Other research was to determine the glycemic index and the glycemic load of 5 South Pacific foods (Plantain (*Musa AAB*), tannia (*Xanthosoma sagittifolium*), roti or chappati, homemade pancake, and Lees cabin crackers) have moderate GI values ranging from 59 to 68. The Glycemic Load (GL) for cabin biscuit was the highest **(Lako et al., 2004)**. Based on sensory test the best treatment was obtained from the proportion of *Xanthosoma sagittifolium* flour: black soybean flour at 70:30 with 2% agar was liked by panelists **(Kasih and Murtini, 2017)**. Biscuits are an instant food that is consumed mostly because of the appetizing, ready to be consumed, the cost is also cheap and the availability of short time and biscuits can make a significant contribution to the daily intake of cereals. However, in an era of increasing functional food popularity, new demands have been set for various categories of snacks including biscuits that can maintain the traditional

nutritional aspects of food and show additional health benefits **(Vujić, Vitali Čepo and Vedrina Dragojević, 2015; Briliannita and Mustika Matto, 2020)**.

Scientific hypothesis

Hypothesis: biscuits with the addition of ants nest tuber and Belitung taro are significantly different in the sensory properties, nutritional value, and antioxidant activity of a variety of biscuits compared to biscuits without addition.

MATERIAL AND METHODOLOGY

Main Material

The main material used in this study was Belitung taro (*Xanthosoma sagittifolium*) and ant nest tubers (*Hydnophytum formicarum*) from the distributor in Sorong City in West Papua, Indonesia (Figure 1 and Figure 2).

Another material was tropicana slim sugar, salt, Max Crimer, Blue Bland Cookies in Mega Mall, The West Papua Indonesia.

Chemical material used in this study was (1) nutritional analysis: protein katalis, H_2SO_4, aquades, borat, BCG asam-MR, NaOH, Na2S2O3, HCl 0.02 N, petroleum ether, buffer HCl-(KC1) bisa pH 4.5, asam acetate pH 1, methanol-PA (2): DPPH analysis: 0.5 mM, methanol, dan vitamin C, vitamin E in Chemix Pratama Yogyakarta.

Chemical analyses of the Biscuits

The chemical composition of fresh biscuits (as prepared on the day of baking) was determined according to the methods of the Association of Official Analytical Chemists International **(AOAC, 2006)**: the total protein content by the Kjeldahl procedure with nitrogen to a protein conversion factor of 5.7; fat content by the Soxhlet method and ash content by carbonization. The total carbohydrate content was calculated by subtracting the sum of moisture, protein, fat, and ash percentages from 100%.

Preparation of dough and baking of the biscuits

The preparation of dough and baking of the biscuits was based on the study of **Krystyjan et al. (2015)**. Dry ingredients were mixed and then combined with others according to the recipe presented in Table 1. The dough was mixed for 10 min to obtain a homogeneous consistency and then placed into the fridge at ±6 °C for 30 min. The dough was then rolled out and 5 mm thick biscuits with a 60 mm diameter were formed and baked at 200 °C for 12 min. (Figure 3). The biscuits with the ant nest bulb powder addition in amounts of 6% and 7.5% concerning the wheat flour were prepared in the same way as the control sample (biscuits without ant nest tuber powder). The results of all the experiments are given as the average of replicates. Each replicate (biscuit) was obtained from separately prepared batches of dough.

Analysis of antioxidant activity (DPPH Method)

Antioxidant activity by DPPH method was based on **(Sompong et al., 2011; Briliannita, 2017** and **Hetharia et al., 2020)** with modification. DPPH 0.1 M was prepared by diluted 0.0039 g DPPH with ethanol 96% until 100 mL. DPPH solution (1 mL) was mixed with 200 µL aliquot and the absorbance was measured at 515 nm after 30 min incubation. The data was served as mg of vitamin C equivalent (VCE) per L with the help of the vitamin C standard curve (0 – 40 $mg.L^{-1}$).

Sensory analysis of the biscuits

For sensory analyses, the laboratory was equipped with special boxes and fulfilled all the basic requirements stated by SNI 01-2346-2006 (Indonesia National Standard, Organoleptic or sensory testing instructions) and sensory analysis was carried out according to **(BSN, 2006; Pauline et al., 2017)** with modification. The evaluation was performed by an untrained panel of 27 women and 3 men that were between the ages of 18 and 25 from students of the Nutrition Department Of the Polytechnic Of Ministry Of Health Of Sorong. For each sample, and for the same panelist, four repetitions were done. The evaluation was done on a Five-point hedonic scale. The scale and categories were as follows: good = 5, fair = 3 and poor = 1. Evaluated characteristics were color, aroma, taste, texture, and overall acceptance.

Statistical analysis

This study used Statistical Product dan Service Solution (SPSS) version 15.0 (Stat Soft, USA). Results were expressed by average ± standard deviation. The effect of the results was performed using the Kruskal-Wallis test (α = 0.05). The samples of individual varieties were evaluated to each other. Furthermore, all samples of individual varieties of biscuits were evaluated against common biscuit (C001).

RESULTS AND DISCUSSION

Chemical composition of the biscuits

Table 2 shows the content of essential nutrients in biscuits. After analyzing the data obtained it was found that the addition of ant nest tuber powder did not affect the carbohydrates content in biscuits – the amount of which oscillated around 60.55%. There was, however, a small but statistically significant increase in ash and water content and a significant decrease in protein and fat content in those biscuits that had additional ant nest tuber powder at 6% and 7.5%. Such a decrease can come from the fact that the ant nest tuber powder contains three times more protein and four times more ash than the Belitung taro flour that was used for baking.

Significant increase of water content for this study, because one to two water populations are identified for Belitung taro flour and are highly dependent on the temperature and concentration rather than the, showed higher retrogradation tendency. An increase in relaxation times at 75 – 80 °C is observed for flours and starches corresponding well to the amylograph pasting temperatures revealed using the Rapid Visco Analyzer **(Boakye et al., 2019)**. The results of other research show that *Xanthosoma sagittifolium* powder proportion, mixing time, and water addition, significantly affect water holding capacity, oil holding capacity, texture, and protein solubility of TVP (p <0.05). The effect of XSP addition on water holding capacity increase when added to 20% **(Lindriati, Herlina, and Arbiantara, 2018)**.

Table 1 Components used for the preparation of biscuit dough (35.25 g).

Material	C001	C013	C014
Wheat Flour (g)	17.6	17.6	17.6
Taro flour (g)	13.2	13.2	13.2
Ant nests tuber (g)	0	1.06	2.64
Vegetable Crimer (g)	0.39	0.39	0.39
Chiken eggs (g)	1.67	1.67	1.67
Margarine (g)	1.49	1.49	1.49
Baking Powder (g)	0.04	0.04	0.04
Sugar of *Tropica Slim* (g)	0.84	0.84	0.84
salt (g)	0.02	0.02	0.02

Note: C001 – without ant nest tuber powder (0%), C013 – 6% ant nest tuber powder, C014 – 7.5% ant nest tuber powder.

Table 2 Content of nutrient raw, and biscuits.

Nutrient Composition	Belitung Taro Raw	Ant Nest Tubers Raw	C001 without ant nest tuber powder	C013 with 6% ant nest tuber powder	C014 with 7.5% ant nest tuber powder
Water (%wb)	8.18 ±0.00^{a}	13.35 ±0.38^{b}	7.35 ±0.12^{a}	9.24 ±0.07^{c}	9.80 ±0.12^{c}
Ash (%db)	14.18 ±0.11^{a}	4.35 ±0.28^{b}	2.82 ±0.21^{c}	5.36 ±0.16^{d}	6.15 ±0.19^{d}
Protein (%db)	2.91 ±0.02^{a}	0.93 ±0.014^{b}	6.00 ±0.03^{c}	4.13 ±0.06^{d}	4.25 ±0.05^{d}
Lemak (%db)	0.67 ±0.05^{a}	0.62 ±0.15^{a}	23.27 ±0.06^{b}	22.17 ±0.19^{b}	19.03 ±0.007^{b}
carbohydrates (bdf*)	74.05 ±0.08^{a}	80.74 ±0.06^{b}	60.55 ±0.31^{c}	59.08 ±0.16^{c}	60.75 ±0.24^{c}

Note: Parameters in columns denoted with the same letters do not differ statistically at the level of confidence $\alpha = 0.05$. Number of replications n = 4.

Table 3 Sensory evaluation results.

Characteristic	Biscuits		
	C001 without ant nest tuber powder	C013 with 6% ant nest tuber powder	C014 with 7.5% ant nest tuber powder
Colour	3.50 ±0.90^{a}	3.57 ±0.72^{b}	3.26 ±0.83^{a}
Aroma	3.86 ±0.77^{a}	3.60 ±0.96^{a}	3.30±0.95^{b}
Taste	3.90 ±0.71^{a}	4.20 ±0.96^{a}	3.30 ±1.11^{b}
Textur	3.57 ±0.77^{a}	3.83 ±0.74^{b}	3.33 ±0.92^{a}
Overall	3.80 ±0.71^{a}	4.10 ±0.72^{b}	3.53 ±0.82^{a}

Note: (a, b) The values with the same letters in the same row are not significantly different (p <0.05).

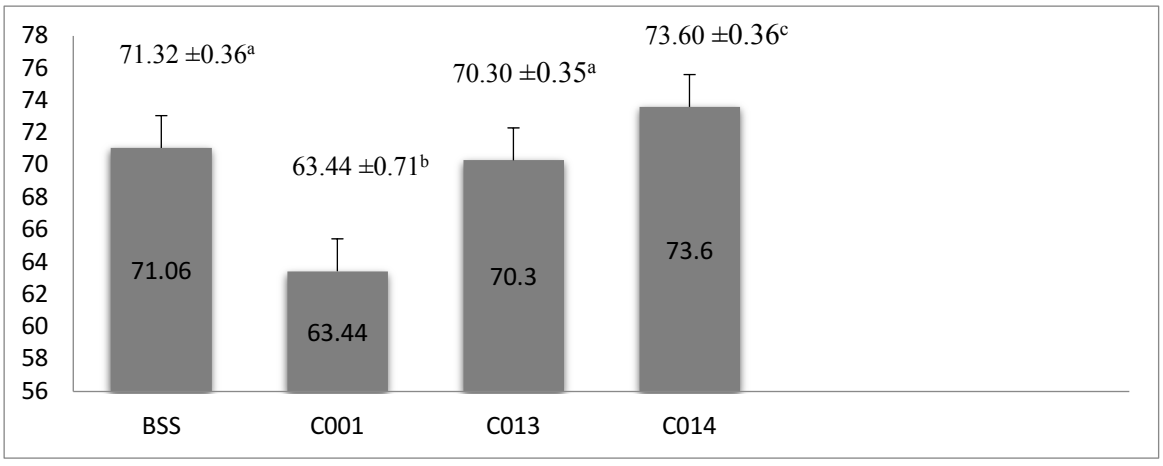

Figure 4 Ability to inhibit free radical by ant nest tuber powder (%) (DPPH Assay).

Based on the research of physicochemical properties of starches of five cocoyam cultivars where starch granule sizes varied significantly in length and width, while amylose content ranged from 11.55% (NCe002) to 33.77% (NXs001). Also, water absorption capacity (21 – 36%), pH (4.8 – 5.3), gelling point (60.5 – 69.5 °C), foam capacity (4.46 – 18.28%), bulk density (0.14 – 1.15 g.mL^{-1}) and swelling power (2.31 – 10.09) varied significantly (p <0.05) among the cultivars. Water content on our research increased **(Falade and Okafor, 2013)**. A significant increase of ash content was not caused by an interaction between Belitung taro flour with ant nest tuber powder. Product ash levels experienced an increase with increasing portions of flour (Belitung taro flour and ant nest tuber powder). This could be because of the high ash content in both materials. Another factor allegedly due to immersion with salt in the preliminary treatment Belitung taro flour. Salt contains minerals including sodium, chloride, and iodine that can be calculated in measuring ash content. Such as in the results of others our research showed that the different sections of *Xanthosoma sagittifolium* cormels were significantly different (p <0.05) in chemical composition. The apical section of all the species had high protein content while the distal section had high levels of ash, fibre, and minerals. Potassium was the most abundant mineral (763 – 1451 µg.100 g^{-1}) with appreciable amounts noted for zinc (17 – 51.1 µg.100 g^{-1}), magnesium (46.7 – 85.0 µg.100 g^{-1}), and phosphorus (41.6 – 63.1 µg.100 g^{-1}) **(Sefa-Dedeh and Kofi Agyir-Sackey, 2004)**.

Antioxidant activity

DPPH Assay

The highest antioxidant activity determined by the DPPH method was biscuit C014 (with 7.5% ant nest tuber powder) 73.60 ±0.36,(p <0.05). The lowest antioxidant activity was determined in biscuit C001 (without ant nest tuber powder) 63.44 ±0.71, p <0.05). Biscuits showed statistically significant differences p <0.05, except sample BSS and biscuit C013, due to their content of ant nest tuber powder difference (Figure 4). Significantly, the ethyl acetate extract was also the most potent antioxidant, showing 83.31% of radical scavenging activity with IC50 8.40 µg.mL^{-1} in the DPPH assay. The other extracts display weak to moderate antioxidative activities, ranging from 28.60 – 56.80% of radical scavenging, α-tocopherol, a positive control, shows antioxidative activity with IC50 6.67 µg.mL^{-1} **(Prachayasittikul et al., 2008)**. Antioxidant activity values ranged from 63.44 ±0.71 to 73.60 ±0.36 in four samples. The other research showed that extracts *Hydnophytum formicarum* could increase lymphocyte proliferation by increasing concentration. There was no chemical content difference observed on extracts *Hydnophytum formicarum*. These extracts contained flavonoids, phenolic, aldehyde/ketone, terpenoids, and tannin **(Darwis, Hertiani and Samito, 2014)**. *Hydnophytum formicarum* showed oxidant activity with IC50 = 7.03 µg.mL^{-1}. *Hydnophytum papuanum* showed oxidant activity with IC50 = 6.19 µg.mL^{-1} **(Makaba, 2017)**.

Sensory evaluation analysis

The sensory evaluation analysis is shown in Table 3. According to the data, biscuits supplemented with 6% ant nest tuber powder obtained a higher total score (4.10 ±0.82) in the sample C013 and the same as in the sample C001 (total score 3.80 ±0.71). The overall assessment allowed the classification of the product to be acceptable (3.53 ±0.82) in the case of the maximum additive (sample C014). When comparing particular sensory characteristics of four biscuits directly, significant differences (p <0.05) were observed between the sample C001 and the sample C014. On this basis, it can be concluded that the applied fortification of biscuits with ant nest tuber powder was possible and gave the desired results, at a level of 6%. Slight differences were noticed between the sample C001 and the sample C014 with a 0% and 7.5% supplementation of ant nest tuber powder. In particular, such characteristics as taste, color, aroma, and texture deteriorated. One of the main factors, as regards the deterioration of the taste of biscuits, was ant nest tuber powder; when used in significant quantities, the biscuits were leaving a crumb biscuit on the tongue. Besides, the color of the product depends on the chemical composition of ant nest tuber powder, which in turn depends on the plant species and environmental conditions **(Agatonovic-Kustrin et al., 2018)**. Thus, the flavonoid, vitamin E, phenolic, aldehyde/ketone, terpenoids, and tannins as well as the reducing sugars introduced to the product with ant nest tuber powder affected its color. Curiously, the darkening of color caused by the addition of ant nest tuber powder was not observed by the assessors.

CONCLUSION

The addition of ant nest tuber powder (7.5%) in the biscuit had an antioxidant activity (DPPH) of 73.60 ±0.36%. According to the sensory evaluation of the biscuits, they were acceptable in characteristics of the color, aroma, taste, texture, and overall. The obtained results have shown that it was statistically significant that panelists will be likely to accept the produced biscuit (p <0.05). The higher the addition of ant nest tuber powder, the higher the antioxidant activity in biscuits and lower the biscuit protein content (p <0.05).

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THE INFLUENCE OF CHOSEN ORGANIC FERTILIZERS ON QUALITATIVE PARAMETERS OF THREE *DAUCUS CAROTA* L. VARIETIES

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ABSTRACT
In rational nutrition, vegetables play an important role due to their high biological and low energy value. The most widespread vegetables in our country belong to root vegetables. They are grown mainly for bulbs, corms, rhizomes, fleshy roots, and hypocotyl tubers. Root vegetables can be eaten raw or cooked. Carrot (*Daucus carota* L.) is a basic representative of root vegetables. For the most valuable components counts beta-carotene – the major component of total carotenoids. This paper evaluates changes in total carotenoids, refractometric dry matter, and gravimetric dry matter in three varieties of carrot (Kamaran F1, Komarno F1, Romosa) grown in soil and climatic conditions ex-situ in Nitra. We have evaluated roots grown in non-fertilized soil, soil after application of manure, horticultural compost, and their combinations. The results show that the variants fertilized with compost and a mixture of compost and manure had the most considerable influence on the synthesis and content of total carotenoids as well as the content of dry matter and refractometric dry matter.

Keywords: total carotenoids; antioxidant; fertilizer; vegetable; quality

INTRODUCTION
Cultural plants are an essential part of civilization. Many of them had originally been harvested as wild plants, later on, cultivated and consciously bred. Vegetables generally represent the juicy parts of plants. They can be grown on arable land, but in the tropics and subtropics, some of the vegetables can be collected from the wild (eg. roots, leaves, etc.). In rational nutrition, vegetables play an important role because of their high biological and low energy value **(Vargová, 2003)**. Vegetables are of great importance for health, especially in terms of the content of vitamins that affect normal metabolism. The beneficial effect of the contained minerals on the acid-base balance in the human body is also significant. Due to their low calorific value, vegetables can be extensively used in a weight-reduction diet. Vegetables, especially when fresh constitute a rich source of vitamins, primarily vitamins A and C. Consumption of vegetables provide many health benefits, such as reducing the risk of heart disease, including heart attack, obesity, diabetes, and stroke. It protects against certain types of cancer and can also reduce the risk of developing kidney stones **(Seljåsen et al., 2012)**. Carrots ranks among the 10 most important vegetable species in terms of cultivated areas' size and production volume indicated in tons **(Simon and Goldman, 2007)**. Carrots produce strong roots with high nutritional value. It contains valuable components such as carotene, thiamine, and riboflavin. It is also a source of carbohydrates, proteins, fat, minerals, vitamin C, and calories **(Yawalker, 1985)**. **Ergun and Süslüoğlu (2018)** state that carrots are primarily valued because of their high beta-carotene contents. In addition to that, the root contains various bioactive compounds including other forms of carotenoids, phenol compounds, and vitamins. Beta-carotene is known as provitamin A and it is known as a strong antioxidant. According to **Oberbeil and Lentz (2003)**, carrots also have a high content of selenium, D, E, and K vitamins, essential oils, lecithin, potassium, calcium, magnesium, iron, copper, phosphorus, iodine, cobalt, and sugars. Among the sugars, the most commonly occurred are sucrose (about 50%), glucose, and fructose **(Šapiro and Raab, 1988)**. Terpenoids are responsible for astringency and bitterness **(Kopec et al., 2010)**. The characteristic taste of carrots is given by their intense sweetness and minimal bitterness **(Simon, 1982)**. Significant amounts of fiber are to be found in the roots. The acid content is low, with malic acid, citric acid, and oxalic acid being the most prominent of the variety of acids contained **(Šapiro and Raab, 1988)**. At present, the total annual yield of carrots in Slovakia has a decreasing tendency compared to the past decade. In 2007 the total annual yield of carrots was 31,817 t per 2,568 ha. Between 2007 and 2008, the yields lightly increased, reaching 37,155 t per 2,562 ha. Afterward, between 2008 and 2010, the total annual yield of carrot mildly lessened up to 34,879 t per 2,454 ha in 2010. However, between 2010 and 2012 total annual crops decreased dramatically to as little as 6,685 t per 250 ha.

Proceeding to 2012 – 2014, the total annual crop of carrots was relatively stable, reaching 6,502 t per 232 ha in 2014. Between 2014 to 2015 though, we were experiencing a slight increase in total annual yield which resulted in 10096 t per 312 ha in 2015. Between 2015 and 2017, the total annual yield decreased again, in 2017 it decreased to 5, 730 t per 185 ha. Average hectare yields in Slovakia in the last decade reached 16 t **(FAOSTAT, 2020)**.

Scientific hypothesis

The concentration of total carotenoids depends on the variety.

More intense usage of fertilizers can support the synthesis of carotenoids, refractometric dry matter, and total dry matter.

MATERIAL AND METHODOLOGY

Characteristics of the research area

The research was carried out in the form of a small-plot experiment under the conditions of the vegetable sampler of the Department of Vegetable Production in the area of Botanical Garden at the Slovak University of Agriculture in Nitra. We used four soil variants, the size of each was 6 x 7 m (42 m²). Monitored varieties are shown in Figure 1 and Figure 2.

Variants

1. variant with the application of livestock manure in the amount of 44 $t.ha^{-1}$ and compost applied in the amount of 44 $t.ha^{-1}$ (MH K).
2. variant with the application of livestock manure in the amount of 44 $t.ha^{-1}$ (MH).
3. variant with the application of mature compost in the amount of 44 $t.ha^{-1}$ (K).
4. variant with non-fertilized soil (control variant) (KON).

By each variant, three experimental trials were carried out.

Characteristics of monitored varieties

***Daucus carota* ´Romosa´ (Rom.)**

A profitable late Berlicum variety is characterized by exceptional external and internal root coloring. The roots are of high quality, cylindrical and 16 – 19 cm long. The flesh is juicy and delicious. This carrot variety reaches a vegetation period of 127 days from sowing to reaping. This variety is recommended for storage. It is suitable not only for storage but also for industrial processing. When densely sown, it is also suited for direct consumption **(Bejo, 2016)**.

Figure 1 Examined carrot varieties, sorted by variety and variant.
Note: left: Kamaran F1, Komarno F1 and Romosa.

Figure 2 Examined carrot varieties.
Note: from the left – Kamaran F1, Romosa and Komarno F1.

***Daucus carota* ´Kamaran F1´ (Cam.)**

This is a late and highly productive hybrid of the Berlicum and Flakkee variety. It forms dull-ended roots up to 26 cm long. It has excellent external and internal coloring. The roots weigh 300 – 500 grams and have a diameter of 2.5 – 4.5 cm. Carrots of this variety are suitable for long-term storage. The vegetation period of Kamaran F1 variety in our climatic conditions reaches 135 days. It can accumulate less nitrate and is suitable for industrial processing and storage **(Bejo, 2016)**.

***Daucus carota* "Komarno F1" (Kom.)**

This variety of carrots is a late and very fruitful Flakkee type variety foremost suited for industrial processing. Komarno F1 variety is typical with high yields and high dry matter content. Its typical smooth conical roots with strong and healthy leaves of dark green color are very resistant to fungal and bacterial diseases. The roots have intense dark red coloring and weigh 200 to 500 grams. Vegetation time for this variety of carrots in our conditions is 161 days. This variety is excellent for freezing, production of carrot juice, and wherever an intense dark color of the final product is required. The variety is also suitable for storage **(Bejo, 2016)**.

Measurement methods of selected qualitative materials

Total carotenoid content

The extraction of samples has been done at the Laboratory of Beverages, AgroBioTechResearch Center of SUA in Nitra. The measurement of total carotenoid content was realized in the laboratory of the Department of Vegetable Production of SUA (Slovak University of Agriculture) in Nitra. The content of total carotenoids was assessed by spectrophotometric measurement of substance absorbance in petroleum ether extract on Spectroquant® Spectrophotometer Pharo 100 at 445 nm wavelengths **(Hegedűsová et al., 2015)**. Calibration was not performed due to the calculation of the total carotenoid content, which was performed according to the average specific absorbance ε. The measurement was taken three times. Total carotenoid content was recalculated according to the relationship reported by **Biehler et al. (2010)**.

Determination of refractometric dry matter

The refractometric dry matter was measured in triplicate by using a refractometer (type CRUESS DR201-95).

Determination of dry matter

The dry matter content was determined by using a gravimetric weighing method. The analyzed plant material was dried at 105 °C up until the weight became stable.

Statistical analysis

Statistical analysis was performed by using Statgraphic Centurion XVII (StatPoint Inc. USA). The obtained results were evaluated by analysis of variance (ANOVA) and the average values were tested by LSD test at the significance level of 95%.

RESULTS AND DISCUSSION

Total carotenoid content

Carotenoids are an important group of biologically active compounds attributed to a wide range of health benefits. These are natural pigments that occur mainly in vegetables but also in other natural resources such as fungi, algae, microorganisms, crustaceans, fish, birds but also mammals **(Kulczyński a Gramza-Michałowska, 2019)**.

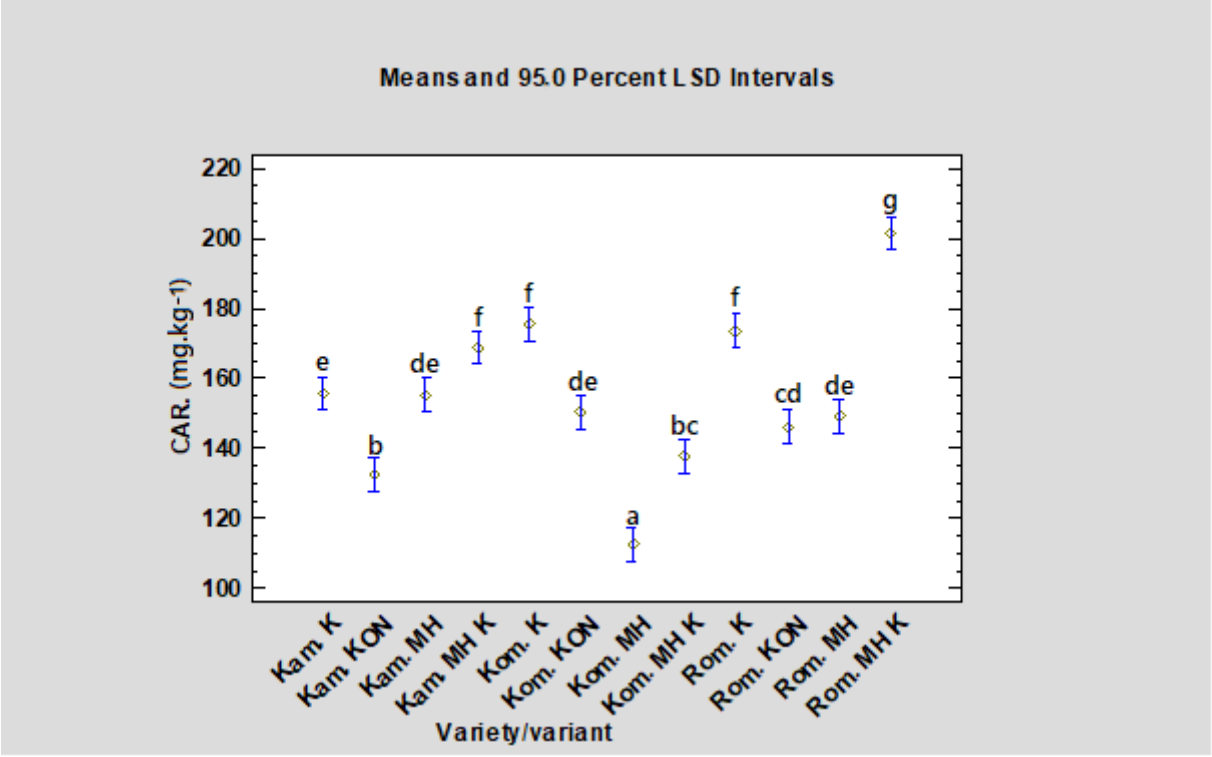

Figure 3 Total carotenoids content (mg.kg^{-1}) in varieties Kamaran F1, Komarno F1 and Romosa according to the variants. Note:The values in the columns with different letters are significantly different from each other.

Based on our results (Figure 3), it is possible to find the highest content of carotenoids in the Romosa variety, namely in the Rom variant MH K (201.32 mg.kg^{-1}). Subsequently, a decreasing trend was observed in the following order: Rom. K by 14% (173.62 mg.kg^{-1}), Rom. MH by 26% (148.96 mg.kg^{-1}) and Rom. KON by 27% (146.03 mg.kg^{-1}). The same findings concerning the variety's particularities were detected in the Kamaran F1 variety. The highest content of carotenoids was recorded in variant Kam. MH K (168.91 mg.kg^{-1}). As for other variants from the Kamaran F1 variety, a decreasing trend can be observed in the following order: K by 7% (155.84 mg.kg^{-1}), Kam. MH by 8% (155.38 mg.kg^{-1}) and Kam. KON by 21% (132.66 mg.kg^{-1}) in terms of carotenoid content determination. Different findings were noted for the Komarno F1 variety. The highest content of total carotenoids in this variety was recorded in the variant Kom K (175.64 mg.kg^{-1}). In other variations of Komarno F1 variety was detected a lower content of total carotenoids decreasing in the following order: Kom. KON by 14% (150.18 mg.kg^{-1}), Kom. MH K by 22% (138.08 mg.kg^{-1}) and Kom. MH 36% (112.62 mg.kg^{-1}). Miękus et al. (2019) and **Amorim-Carrilho et al. (2014)** report that the total carotenoid content of carrots is generally 160-380 mg.kg^{-1} of fresh matter. **Fikselová et al. (2008)** report that the carotenoid content of most varieties ranges from 60 – 120 mg.kg^{-1} of fresh matter, but some varieties have a carotenoid content of up to 300 mg.kg^{-1} of fresh matter. The varieties examined in our study can certainly not be evaluated as highly above average regarding their content of carotenoids. **Kaur and Sogi (2016)** report a total carotenoid content of 324 mg.kg^{-1} while **Saha et al. (2016)** in their experiments set the highest content at 144 mg.kg^{-1}. **Kiraci and Padem (2016)** observed purple varieties of carrots and found out that the content of beta-carotene (the main constituent of carotenoids in carrots) is 117 – 149 mg.kg^{-1}. A study by **Santos and Simon (2006)** confirms that the content of carotenoids is genetically determined for individual varieties**. Smoleń and Sady (2009)** revealed no significant effect of N-fertilization on carotenoid concentration in carrots. Our research has refuted this claim. However, obtained results confirm the findings of **Evers (1989)**, who claims in his study that variants treated with P and K or NPK fertilizers were characterized by a higher carotene content in the roots.

Hochmuth, Brecht, and Bassett (1999) studied the total carrot root yield and the content of total carotenoids and sugars. They found the levels of N fertilization maximizing carrot root yield (in tonnes) also maximized carrot quality in terms of sugar and carotenoid levels. **Kovács et al., (2012)** verified the use of compost, mineral fertilization, and bacterial fertilization in carrot cultivation. The highest content of total carotenoids in the roots was detected in variants with compost. Recent studies have shown that the content of total carotenoids in carrot roots can change completely when the roots are exposed to light. Illuminated roots have chloroplasts with high lutein levels instead of beta-carotene-rich chromoplasts found in roots below the ground **(Rodríguez-Concepción and Stange, 2013)**. From carrot production with an emphasis on the production of the high content of beta-carotene, it is important to choose the appropriate agrotechnical.

Dry matter content

The results (Figure 4) show that the highest dry matter content was determined in the Kamaran F1 variety, namely in the Kam. K (14.16%) variant.

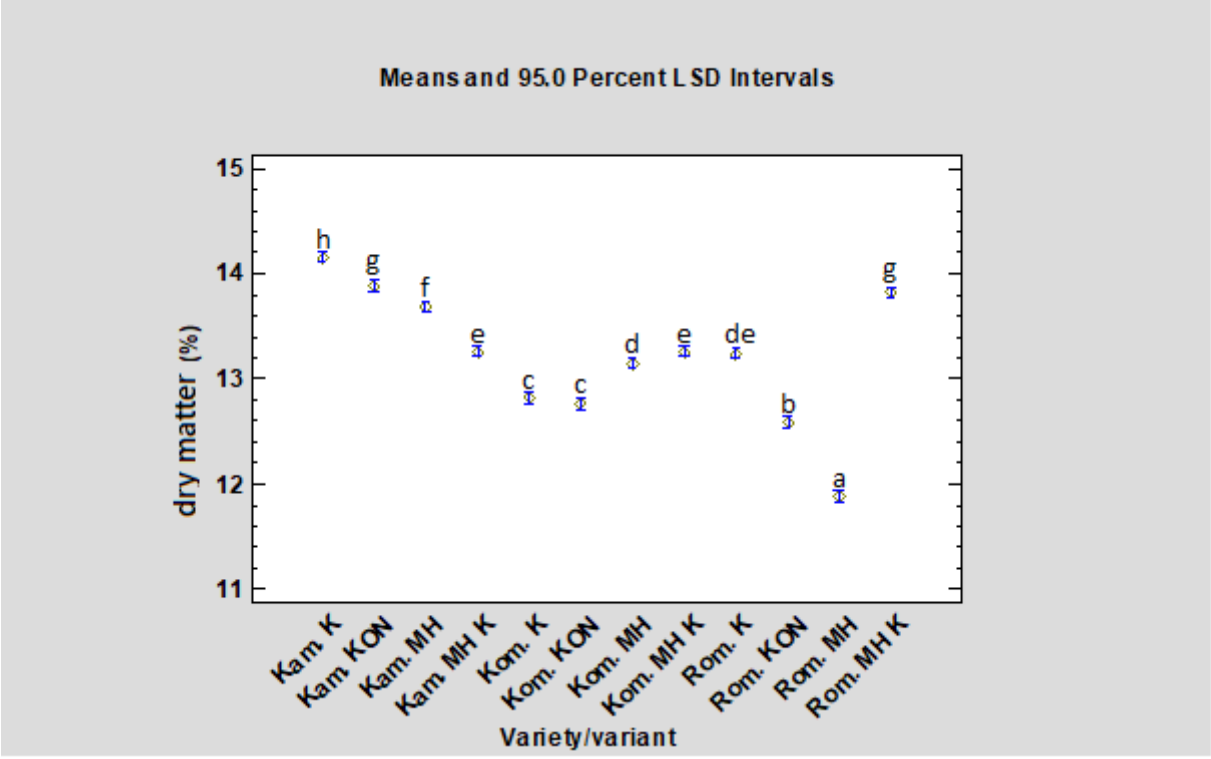

Figure 4 The dry matter content in (%) in varieties Kamaran F1, Komarno F1 a Romos aaccording to the. Note: The values in the columns with different letters are significantly different from each other.

In the case of the other variants (Kamaran F1 variety), the decreasing trend in the order of Kam. KON by 2% (13.88%), Kam. MH by 3.3% (13.69%) and Kam. MH K 6.4% (13.26%) can be noted in the dry matter contents. In the Romosa variety, other findings were detected in terms of variation effects. The highest dry matter content was determined in the Rom. MH K (13.82%) variant. In the case of the other variants (Romosa variety), the decreasing trend in the order of Rom. K by 4.2% (13.24%), Rom. KON by 8.9% (12.59%) and Rom. MH by 13.97% (11.89%) can be observed in the dry matter contents. The highest dry matter content of Komarno F1 was determined in the variant Kom. MH K (13.26%). In the case of other variants (Komarno F1 variety), the decreasing trend in the order of Kom. MH by 0.83% (13.15%), Kom. K by 3.3% (12.82%) and Kom. KON by 3,7% (12,77%) can be stated in the dry matter content. Variants fertilized with compost and a mixture of compost and livestock manure had the highest impact on the dry matter content. **Bach et al. (2015)** reported that the dry matter content of most varieties varied from 11.1 to 13.7 $g.100^{-1}$ g of fresh weight. However, the authors add that in 2009, the dry matter content was significantly higher ($p \leq 0.05$) than in 2007 and 2008. In comparison with Bach, a relatively high average dry matter content was found in our study. **Dhillon et al. (2016)** reported that the dry matter content of the carrot varieties studied ranged from 6.68% to 8.70% (average 7.66%). Compared to Dhillon, we again detected a significantly higher percentage of dry matter. However, the varieties examined by us cannot be assessed as highly above average concerning their dry matter content. **Gopalan, Rama Sastry, and Balasubramanian (1991)**, **Longvah et al. (2017)**, and **Fanlégué et al. (2018)** agree with us that the moisture content of carrots varies between 86 and 89%. **Kaur and Sogi (2016)** determined a 10% dry matter.

Refractometric dry matter

The highest refractometric dry matter content was determined in the Kamaran F1 variety (Figure 5), namely in the Kam. variant K (11.98 °Brix). We observed a decreasing trend in the contents of the refractometric dry matter in other variants of Kamaran F1 variety with KON declining by 5.1% (11.37 °Brix), Kam. MH by 6.5% (11.2 °Brix) and Kam. MH K by 10.02% (10.78 °Brix). Different findings emerged from the measurements of the Romosa variety.

The highest refractometric dry matter content was determined in the Rom. variant MH K (11.22 °Brix). Other variants of the Romosa variety showed a decreasing trend in the following order: KON by 3.48% (10.83 °Brix), Rom. K by 4.46% (10.72 °Brix) and Rom. MH by 12.84% (9.78 °Brix). Slightly different conclusions can be drawn from researching the Komarno F1 variety. The highest content of refractometric dry matter was determined in variant Kom. MH K (10.63 °Brix). In the case of other variants (Komarno F1 variety) a similar decreasing trend was recorded in the refractometric dry matter content which declined in the following order: MH by 0.28% (10.6 °Brix), Kom. KON by 6.21% (9.97 °Brix) and Kom. K by 7.06% (9.88 °Brix). Variants fertilized with compost and a mixture of compost and manure had the highest impact on the refractometric dry matter content. **Valšíková et al. (2013)** report that the average refractometric dry matter content of fresh carrot roots was 7.6 °Brix. Authors also recorded that after 14 days of package-free storage in a laboratory the refractometric dry mater values increased to 14.8 °Brix. In comparison with Valšíková, we detected higher average values of refractometric dry matter contents in our research. **Seljåsen et al. (2013)** state that the refractometric dry matter content values in carrots varied from 45 – 72 $mg.g^{-1}$ of fresh matter (60%) depending on the variety.

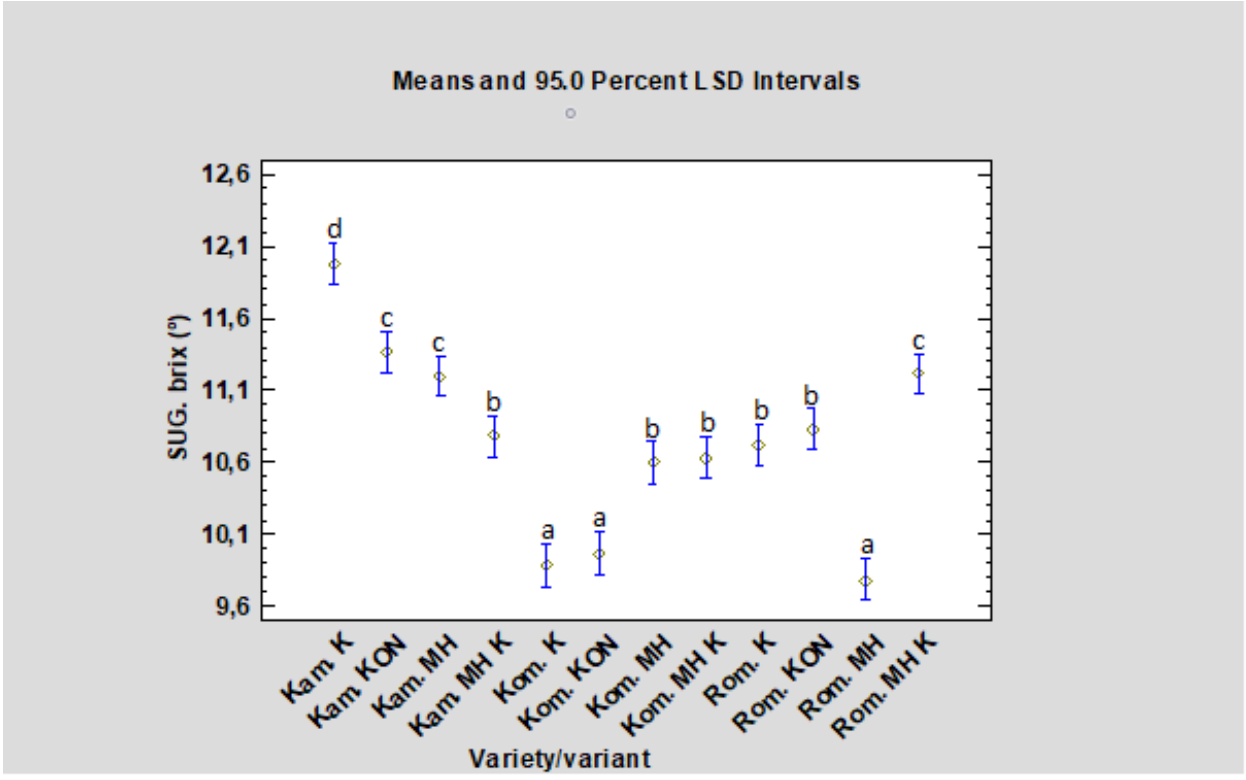

Figure 5 Refractometric dry matter content in Brix (°) in varieties Kamaran F1, Komarno F1 a Romosa according to the variants. Note: The values in the columns with different letters are significantly different from each other.

Machewad et al. (2003) claim that carrot root is considered one of the tastiest and juiciest roots and the total soluble solids content mean value reaches approximately 12 °Brix. **Saha et al. (2016)** detected results stretching from 6.7 to 10.48 °Brix. Similar findings (6 – 8 °Brix) were published by **Jabbar et al. (2015)**; **Gills et al. (1999)** reported values ranging 8.6 – 10 °Brix; **Holley et al. (2007)** 7 – 10.5 °Brix; **Nadulski et al. (2014)** 8.2 °Brix; **Evrendilek and Ozdemir (2019)** reported values ranging from 7.2 to 9 °Brix; and **Santana-Gálvez et al. (2019)** came with the resulting values of 8.1 °Brix. Comparing these to the results of our research, we detected lower values of carrots' refractometric dry matter. **Smoleń and Sady (2009)** do not hold coincident opinions on the impact of fertilization (foliar) on refractometric dry matter content. In their experiments, the refractometric dry matter content increased in some cases, while decreased in others.

CONCLUSION

In four soil variants model varieties of carrots (Romosa, Kamaran F1, Komarno F1) were evaluated in terms of qualitative parameters (total carotenoids, dry matter, and refractometric dry matter). The study of total carotenoid content in carrot varieties shows that the most significant effect on the synthesis and carotenoid content was detected in the variants fertilized with compost and a mixture of compost and manure. Averaging the results from all four variants, the best source of carotenoids in terms of varieties was measured in the Romosa variety (167.48 $mg.kg^{-1}$). The lower content of total carotenoids was found in Kamaran F1 (153.19 $mg.kg^{-1}$, 9% lower) and Komarno F1 (144.13 $mg.kg^{-1}$, 14% lower) varieties. The variants with compost and a mixture of compost and livestock manure had the highest influence on the content of dry matter. The Kamaran F1 variety was found to have the highest average dry matter content (13.75%). Lower dry matter content was found in Romosa variety (12.88%, by 6.33% lower) and Komarno F1 variety (13%, by 5.46% lower). What regards the refractometric dry matter, the highest effect on the varieties had variants fertilized with compost and a mixture of compost and livestock manure. Averaging the results of all the variants for the specific variety, the highest refractometric dry matter content was detected by the Kamaran F1 variety (11.33 °Brix). A lower dry matter content was found in the Romosa variety (10.64 °Brix, lower by 6.09%) and Komarno F1 (10.27 °Brix, lower by 9.36%).

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A CASE STUDY COMPARING DISTILLATION TECHNOLOGIES FOR PLUM PALINKA PRODUCTION

Péter Korzenszky, Gábor Barátossy, László Székely, Gábor Géczi

ABSTRACT

Palinka production has a long tradition in Hungary and the neighboring countries. Previously, the fruit distillate was produced exclusively using the traditional Pot-Still Double Distillation (PSDD) technology. This distillation method means, in practice, a simple fractional distillation repeated twice. However, in other industries, such as the petroleum industry or the pharmaceutical industry, a continuous, so-called repeated distillation procedure is used (RCDS – Rectification Column Distillation Systems). In the production of palinka, the latter procedure has gained more and more ground in recent years, thus displacing the traditional technology. In the territory of today's Hungary, there are more than 16,000 registered private palinka distillers. However, based on public databases, it is not possible to know the proportion of the two different palinka making processes used in palinka production. The two processes differ to a large degree. The amount of hearts obtained using the continuous operation plate rectification column (RCDS) is lower, while its alcohol content is very high: 75 – 90 vol%, depending on the fruit. On the other hand, when using the traditional pot-still double distillation (PSDD) method, the amount of hearts is higher, but its alcohol content is lower (60 – 70%). The continuous procedure, also called single-stage, is faster. This is one of the reasons for its popularity because it makes production more economical. The objective of our research was to find out whether a significant difference could be detected between the two plum palinkas produced using the two different distillation technologies, based on current legal requirements. Our research also included sensory testing to determine whether consumers could distinguish between the products manufactured in different ways. Our analyses were carried out in 2019 in the accredited laboratory of the National Food Chain Safety Office and among the students and staff of the Gödöllő campus of Szent István University.

Keywords: plum palinka, distillation, pot-still double distillation, rectification column

INTRODUCTION

According to the current regulation, only alcoholic beverages prepared from fruit produced in Hungary, by fermentation and distillation and with an alcohol content between 37.5% and 86% (V/V%) can be called palinkas **(László, Hodúr and Csanádi, 2016; Panyik, 2018)**. The name palinka can also be used for beverages produced in four Austrian provinces from apricots. However, distillation is also used in other countries to produce alcoholic products: brandy, spirit schnaps, obstbrand (fruit-based), vodka (corn, potatoes), whiskey (grain), borovička (juniper), cachaca (sugar cane juice), rum (cane sugar), tequila (blue agave), mezcal (agave), poitin (barley malt), baijiu (sorghum, rice). Alcoholic drinks, made by fermentation and distillation from fruits, mainly from plums, are very popular in Europe, primarily in Slovakia (*Slivovica*), the Czech Republic (*Slivovice*), Poland (*Sliwowica*), Serbia (*Prepečenica and Sljivovica*), Romania (*Tuica*) and Hungary (*Szilvapálinka*) **(Portugal, et al., 2016; Śliwińska et al., 2016; Satora and Tuszyński, 2008; Zheng et al., 2014)**.

Due to the technology, the quality of palinka is influenced by three well-distinguishable stages: the fruit itself (its variety, state of ripeness, and date of harvest); the mashing procedure, and, finally, the distillation technology. Storage and consumption habits may also be influencing factors, but experts agree that the three areas listed are dominant. In the course of our research, distillation technology has been analyzed. Distillation is a separation technology process in which the volatile components that enter the vapor phase during the evaporation are separated from the liquid phase. This is followed by the condensation of the generated vapors and re-liquefaction. The composition of the condensate formed after recooling the vapors that formed during the distillation of the mash is different than that of the mash since the mash does not contain only volatile compounds. The different components are found in higher concentrations in the distillate than in the mash, depending on their volatility. Distillation has a dual purpose during the brewing of palinka. On the one hand, the extraction of the alcohol content of the mash, and on the other hand, the separation of undesirable volatile components present in

the mash from the precious hearts, by including them in the heads and the tails **(Nagygyörgy, 2010)**.

The traditional Hungarian method is considered to be the double distillation performed in pots, and this is commonly called pot-still technology. By definition, pot-still technology is brewing in an apparatus that has a pot with a volume of no more than 1,000 liters. The pot-still technology begins with the distillation of the mash that has a relatively low alcohol content (2 to 10%). The first distillate (brute alcohol) has an alcohol content of 15 to 30%, depending on the fruit and the apparatus. The second step is the refining of the brute alcohol, that is, the increase of the alcohol concentration to 60 – 70%. At the same time, the heads and the tails are also separated. PSDD (Figure 1a) in the breweries is usually carried out in two separate pots, mainly due to excise regulation and economic operation reasons. However, technically, the second distillation can be carried out in the same pot, but this is most typical of home brewing.

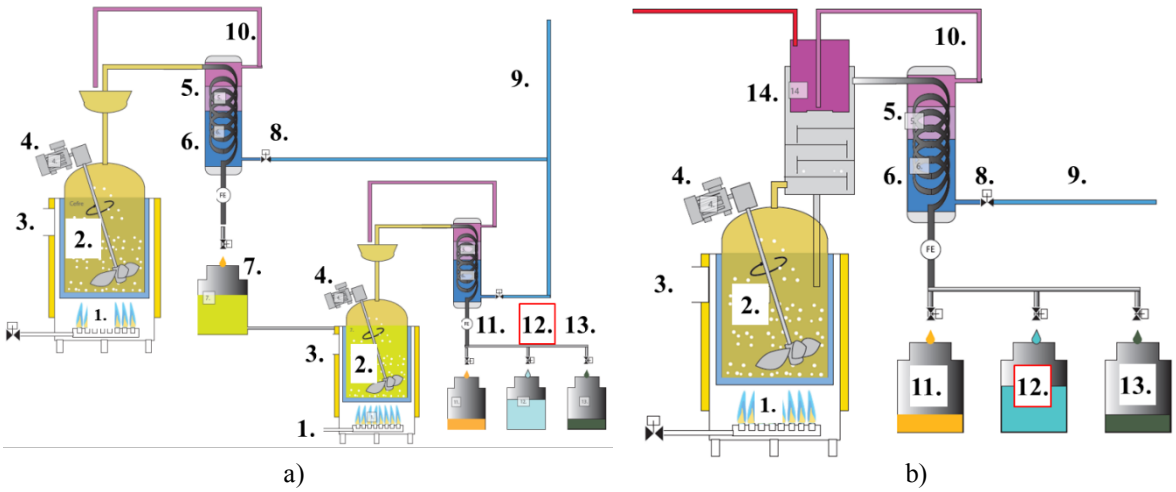
The other technology, which is gaining more and more ground due to Austrian and German influences, is continuous distillation based on column or tower apparatuses (RCDS). In the case of the RCDS technology, multiple distillations can occur in the distillation column. The operating principle is based on having the upward flowing vapors containing volatile substances meet a downward liquid stream while ensuring an adequate exchange of heat and material. The meeting of the vapors and the reflux liquid takes place on the plates in the column. Going up, the alcohol and other volatile component content of the vapor increases and, after condensation of the vapor, a distillate with an ethyl alcohol concentration of 70 to 90% can be obtained. (Figure 1b). In tower systems, depending on the design, 4 to 6 rectifications are carried out, but the separation of the heads and the tails are realized here as well in the final distillate (condensate) **(Nagygyörgy, 2010; Balcerek et al., 2017, Géczi, Korzenszky and Nagygyörgy, 2018)**.

From a palinka brewing point of view, one of the most important points of distillation is the sharp separation of the heads. According to the theory, the amount of the heads is appropriate if the highest possible amount of aroma, characteristic of the fruit, is transferred to the hearts, but unfavorable components, mainly ethyl acetate, are only included in the hearts in such small amounts that they do not cause sensory faults. After the separation of the heads, distillation is continued by the collection of the hearts. The ethyl alcohol content of the vapor decreases continuously and, at the same time, the concentration of the necessary aromas decreases as well, and if distillation is continued, the condensate will have an undesirable, unpleasant sour taste. So the characteristics of the heart are influenced not only by the distillation apparatus but also by the selection of the heads and tails cut points. Palinka suitable for consumption can then be obtained by rest and dilution with softened water **(Nagygyörgy, 2010)**.

To improve the quality of palinka, research into palinka distillation is ongoing, with the number of papers increasing significantly in recent years, although being still less than the amount of scientific publications investigating the quality of beer and wine products.

The determination of the cut points is clearly of great importance, and a numerical method to predict cut-points was developed by **Gössinger et al. (2012)** of Austria for apple distillates. For the more accurate identification of the cut points, research has been focusing on the determination of the volatile components characteristic of the fruit. **Rodriguez-Solana et al. (2018)** performed the analysis of fig distillates popular in Mediterranean countries. 130 volatile compounds were identified in the fig distillates, including, as common constituents, ethyl decanoate, ethyl octanoate, and ethyl dodecanoate, the aldehydes benzaldehyde and furfural, the monoterpene limonene, and the norisoprenoid β-damascenone. Knowledge of the volatile components also allows for objective control of the market, which is a part of the fight against counterfeiting as well.

Claus and Berglund (2005) showed in the case of a tower system technology that the plate number and dephlegmation (reflux) play significant roles. The concentrations of ethanol and related compounds, such as methanol, acetaldehyde, ethyl acetate, 1-propanol, and



a) b)

Figure 1 The two typical distillation technologies.
Note: a) Schematic representation of the pot-still technology (PSDD); b) Schematic representation of the rectification column technology (RCDS). 1 – heating; 2 – mashing; 3 – flue gas discharge; 4 – stirring; 5 – condensation; 6 – cooling; 7 – first distillation; 8 – flow measurement; 9 – cooling media IN; 10 – condenser outlet; 11 – heads; 12 – hearts; 13 – tails; 14 – distillation tower.

isoamyl alcohol in the final product were affected by the plate number.

The effect of dephlegmation was also investigated by **Rodríguez-Bencomo et al. (2016).** The results showed that a high reflux ratio at the beginning of the distillation is a good way to reduce the amount of unpleasant compounds in the hearts. **Nagygyörgy (2016)** paid great attention to the research of the effect of dephlegmation. It was determined by him that dephlegmation is primarily determined by the surface of the dephlegmator, the temperature of the vapor tube, and the intensity of heating.
In addition to research into cut points and components that determine advantageous quality, there has been a strong focus on studies investigating the legal background of palinka production and the effects of changes in it **(Zsótér and Molnár, 2015)**. **Deák et al. (2010)** stressed the importance of ethyl carbamate detection in their article. Ethyl carbamate (EC, urethane, $C_2H_5OCONH_2$) is a genotoxic carcinogen and is regularly found in fermented foods, including alcoholic beverages **(Monakhova, Kuballa, Lachenmeier, 2012).** The target value for ethyl carbamate according to the recommendation of the European Food Safety Authority (EFSA) is 1 $mg.L^{-1}$. For a rapid determination of the components, **Śliwińska et al. (2016)** drew attention to the suitability of the electronic nose.

The importance of the cut points and product components is not disputed by anyone, and many professionals see technology development as the solution. A pervaporation membrane technology for the production of apple palinka was developed by **Molnár, Márki and Vatai (2016)**, which is energetically more efficient than the traditional pot-still technology.

García-Llobodanin et al. (2008) examined the concentration and quality of the raw material placed in the still and, regarding pear distillates, found that using natural pear juice does not offer any benefit when compared to using concentrated pear juice from Blanquilla variety pears.

Balcerek et al. (2017) investigated the effect of pot-still and rectification column distillation technologies on the hearts, namely the distribution of the volatile matter content and the concentrations of undesirable compounds (methanol, hydrogen cyanide, ethyl carbamate) in plum palinka. Irrespective of the distillation method used, the heads contained mainly aliphatic aldehydes, acetals, and esters, as well as higher alcohols (1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, and 3-methyl-1-butanol). Increasing the alcohol concentration in the hearts from 70% to 90% resulted in the gradual decrease in the concentration of all detected volatile compounds. Compared to the pot-still technology, single-stage distillation (tower system) resulted in hearts with lower concentrations of acetaldehyde and benzaldehyde. In the case of the pot-still technology, a statistically significant increase in the amount of methanol and ethyl carbamate in the hearts was observed.

Technological, as well as tourism issues, are discussed by **Harcsa (2017a)** and **Harcsa (2017b)**, in his research aimed at the comparison of the energy consumptions of the two different systems (pot-still and tower).

Kovács et al. (2018) stressed that the quality of fruit-based alcohol content varies from year to year; therefore, it is necessary to identify the vintage of distilled alcoholic beverages. They found that three ingredients are associated with the vintage, regardless of the type of fruit: propanol, butanol, and ethyl propionate.

It was mentioned in the introduction that some of the most popular distillates in neighboring countries are made from plums, and based on this it is understandable that plum palinka is a popular research subject. **Jakubíková et al. (2019)** showed differences between the origin and harvest time of raw materials by examining the phenol and anisole compounds of plum palinkas. **Satora et al. (2017)** examined the quality of distillates prepared from four varieties of plums and found that the aroma of the samples is determined by six compounds (ethyl dodecanoate, benzyl acetate, methyl cinnamate, 1-heptanol, α-terpineol and benzothiazol).

Pecić et al. (2012) found that the ageing of Serbian plum palinka in wooden casks not only improves organoleptic characteristics, but also changes the total polyphenol content and contributes to other important health properties, such as the increase in their antioxidant capacity.

As early as 1980, **Ismail, Williams and Tucknott (1980)**, identified 54 constituents based on the gas chromatographic and mass spectrometric analysis of volatile compounds in the distillate of fermented plum leaves, including 11 hydroxy compounds, 1 acid, 30 esters, 4 carbonyl compounds, 3 lactones, and 5 acetals. Thirty of these compounds had not been previously reported in fermented plum products. Benzaldehyde, linalool, methyl cinnamate, and y-decalactone, based on gas chromatographic odor assessment, are believed to contribute significantly to the plum character of this beverage. However, in 1982, **Velíšek et al. (1982)** pointed out that, in the Czechoslovakia of the time, when examining the volatile taste components of plum palinka, higher aliphatic aldehydes (nonanal and some others), 2-undecanone, benzaldehyde, damascenone, benzyl acetate, ethyl phenylacetate, phenyl ethyl acetate, ethyl 3-phenyl propionate, methyl cinnamate, and ethyl cinnamate were the most significant contributors to the typical plum palinka aroma.

Sádecká et al. (2016) used synchronous fluorescence spectroscopy, combined with principal component analysis (PCA) and linear discriminant analysis (LDA), to distinguish plum distillates according to their geographical origins. A total of 14 Czech, 12 Hungarian, and 18 Slovak plum distillate samples were used.

Jurica et al. (2016) investigated the occurrence of phthalates during the production of plum distillates and in the plum distillate final product manufactured by registered producers in five European countries using gas chromatography-mass spectrometry (GC-MS). As the distillation process progressed, a decreasing trend was observed for the mean values of diethyl phthalate (DEP), diisobutyl phthalate (DiBP), and di-n-butyl phthalate (DBP).

Satora and Tuszyński (2008) found that homemade Polish plum palinka usually contained more ethanol (64.7 – 72.5 vol%), methanol (5.59 – 8.74 $g.L^{-1}$ AA) and butanol (32 – 335 mg. L^{-1} AA) and less isobutanol (406 – 491 mg. L^{-1} AA). The results showed that plum palinkas produced in the Łacko area are characterized by similar and original

chemical compositions, which come mainly from spontaneous fermentation, as well as the traditional production technology.

The importance of plum palinka in Hungary is demonstrated by the fact that in 2013 it was included on the list of Hungarikums. Hungarikums are products, foods, or values that represent Hungarian traditions with their properties, uniqueness, specialty, and quality **(Harcsa, 2018)**.

Fodor, Hlédik and Totth (2011) demonstrated that the popularity of palinka is on the rise, but consumers are choosing well-known brand names. The role of the regions can also be observed in Hungary in connection with plum palinka, indicating a certain quality: plum palinka of Békés and Szatmár **(Kassai et al., 2016)**. Food safety is becoming increasingly important among consumers. According to a survey of Slovak researchers, in Slovakia, 84% of respondents buy higher-quality foods **(Nagyová et al., 2019)**.

A large scale questionnaire survey was conducted by **Szegedyné Fricz et al. (2017)** to evaluate consumer behavior. Their conclusion was, among other things, that a significant proportion of consumers do not have even the slightest knowledge regarding the production of palinka.

Scientific hypotheses

Hypothesis No. 1: The instrumentally measurable content values, based on legal obligations, of final products prepared by various distillation technologies from the same batch of raw materials with identical preparation procedures are different.

Hypothesis No. 2: The final products prepared by various distillation technologies from the same batch of raw materials with identical preparation are different based on sensory evaluation.

MATERIAL AND METHODOLOGY

Materials

The raw material used in the study was an 18 Brix% (g sugar/100 g mash) mash made from President (*Prunus domestica President*) and Stanley (*Prunus domestica Stanley*) plums harvested in mid-September 2018. President plum is a slightly elongated, very large (45 – 55 g), hard, freestone, sweet variety with a purple-red skin, yellow flesh, and a pleasant taste. Stanley plum is one of the most widespread varieties in the world, it is large (diameter 34 – 36 mm), its peel is dark blue, strongly waxy, the flesh is yellowish-green, tasty, juicy, freestone. (Figure 2) Due to the unusually warm weather, the ripening period of the two varieties was longer, making it possible to harvest the required amount of both varieties at the same time at their optimal stage of ripeness.

Preparation

The mashing procedure took place in Bózsva, at the premises of Tiszta Gyümölcs Kft. According to the planned program, 500 kg of pitted plums were fermented at 18 °C in a stainless-steel tank that could be cooled and was equipped with a stirrer for 21 days. At the same time when the precooled raw material was placed in the tank, Lallyzim HC pectin breakdown mixture was added to the raw material. The pH was adjusted (pH = 3.2) with a 10% solution of citric acid, and inoculation was performed by the addition of Uvaferm 228 yeast and Uvavital yeast nutrient.

After the fermentation of the mash was over, the raw material was homogenized by stirring, and then it was divided into two parts. Tower system (RCDS) distillation was carried out at the same site in Bózsva, using the German-made Christian Carl equipment of Tiszta Gyümölcs Kft., and the plum palinka thus obtained was marked I. The location of the pot-still technology was the brewery of the Veresegyház Palinka House, where the distillation was carried out using the Czech-made Kovodel Janca s.r.o. double-still (PSDD) equipment and the final product thus obtained was marked II.

Due to the various distillation processes, the products were available with different alcohol concentrations. In the case of the pot-still technology, the alcohol content of the hearts was 66.9 vol%, while in the case of the column technology it was 84.4 vol%.

The final products were prepared by adjusting the alcohol concentration to the planned value of 44 vol% with the same distilled water.

This way, 24.1 liters of product were obtained during pot-still distillation and 23.5 liters using the tower technology. Analytical tests were performed in February 2019, while sensory evaluations took place in October 2019, when the plum palinka was 1 year old.

President Stanley

Figure 2 The plums included in the study (*Prunus domestica*).

Table 1 The parameters measured in the tests and their measurement methods.

Parameter	Measurement method	Measurement technique	Limit of quantification	Regulatory limit value
Actual alcohol	IR-1:2000	Anton Paar, Alcolyzer	1.00%vol.	37.5% – 86%
Acetaldehyde	Regulation (EC) No 2870/2000*	GC	3.7 mg.L^{-1}	no limit value
Allyl alcohol	Regulation (EC) No 2870/2000*	GC	2.0 mg.L^{-1}	no limit value
Methanol	Regulation (EC) No 2870/2000*	GC	2.0 mg.100cm^{-3}	1200 g.hL^{-1} AA
Propanol	Regulation (EC) No 2870/2000*	GC	1.6 mg.L^{-1}	no limit value
2-Butanol	Regulation (EC) No 2870/2000*	GC	0.7 mg.L^{-1}	no limit value
2-Methyl-1-propanol	Regulation (EC) No 2870/2000*	GC	1.3 mg.L^{-1}	no limit value
n-Butanol	Regulation (EC) No 2870/2000*	GC	1.9 mg.L^{-1}	no limit value
2-Methylbutanol	Regulation (EC) No 2870/2000*	GC	0.8 mg.L^{-1}	no limit value
3-Methylbutanol	Regulation (EC) No 2870/2000*	GC	2.0 mg.L^{-1}	no limit value
Volatile matter	Regulation (EC) No 2870/2000*	Calculated value	2.0 mg.100cm^{-3}	min.: 200 g.hL^{-1}AA
Ethyl acetate	Regulation (EC) No 2870/2000*	GC	1.7 mg.L^{-1}	no limit value
Acetal	Regulation (EC) No 2870/2000*	GC	2.9 mg.L^{-1}	no limit value
Total hydrogen cyanide	MSZ 9589-12:2013	titrimetry	1 mg.100cm^{-3}	7 g.hL^{-1}AA
Ethyl carbamate	CEN/TC 275/WG 13:2012	GC-MS	0.41 mg.L^{-1}	no limit value

Note: *Method III.2 of **Regulation (EC) No 2870/2000** for alcoholic beverages (distillate).

Analytical test

Laboratory analysis of samples was performed by a blind test in the testing laboratory of the Directorate of Oenology and Alcoholic Beverages of the National Food Chain Safety Office, accredited under reg. no. NAH-1-1673/2015, under current legal regulations. 15 parameters were tested for both samples with multiple replicates. The tested parameters, measurement methods, descriptions of the measurement techniques, limits of quantification, and regulatory limit values are given in the following table (Table 1). Sampling and sample preparation was carried out by qualified personnel.

Organoleptic tests were carried out according to Hungarian Standard MSZ 9600:2016 Guidelines for sensory analysis of spirit drinks.

Organoleptic test

Tests were carried out among the students and staff of the Gödöllő campus of Szent István University, in groups of 15 – 25 people, on 18 occasions in October 2019, in the Food Technology and Machines Laboratory of the university. All participants were occasional palinka consumers who participated in the study voluntarily. Test conditions (room temperature, palinka temperature) were the same in all cases so that external parameters did not affect the results.

During the organoleptic tests, samples were marked only with labels suitable for distinction („I" and „II"), test subjects did not know which mark meant what. For the sensory examination, samples with a volume of 2 cl were provided. A total of 341 people participated in the sensory examinations, all of them only once.

In each case, during the sensory evaluation, respondents had to answer the question: „Do you feel a difference between the two samples?" Those who did feel a difference, had to answer the question: „Which palinka sample tasted better?" The questionnaire included a minimal number of demographic questions regarding the gender and age of the respondents.

Statistical analysis

Statistical analysis was performed based on the data obtained during the laboratory tests. The difference in the variance of the two-sample series was checked by the *F*-test. Since there was no significant difference between them two-tailed independent sample *t*-test was used to determine if there is a significant difference between any of the examined parameters. We say that the difference is significant if the corresponding *p*-value $p < 0.05$ holds.

Questionnaires of the sensory examinations were summarized using Excel. As a further analysis, we examined whether the choice of palinka of our sample population depends on gender or age. To this end, contingency tables were applied. The dependencies were examined by using Pearson's χ^2-test, furthermore, Goodman, and Kruskal's λ value was calculated to measure the strength of the association.

Our data analysis was performed using the data analysis module (Analysis ToolPak) of Excel and IBM SPSS 25.

RESULTS AND DISCUSSION

A test report was compiled on the measurement results of the different plum palinka samples analyzed in the accredited testing laboratory. Available data were processed and evaluated using the statistical methods described.

The following table lists the 15 parameters tested by the laboratory and the average values of 10 measurement series for both samples (Table 2). For the anonymous identification of the samples during the laboratory tests, marks of I and II were used. Sample I marked the final product of the tower distillation apparatus, while sample II meant the plum palinka sample made with the traditional pot-still technology.

Based on the statistical analysis it can be stated that our hypothesis No.1 must be rejected because no significant differences ($p < 0.05$) could be detected between the two samples in the statutory palinka quality parameters of the final products prepared from the same batch of raw material and the same preparation using various distillation technologies.

Table 2 Statistical results.

Measured parameter	Expected value of sample I	Expected value of sample II	p value of *F*-test	p value of *t*-test
Actual alcohol [%]	44.08 ±0.23	44.00 ±0.34	0.2670	0.5461
Acetaldehyde [mg.L^{-1}]	69.1 ±4.56	64.3 ±5.14	0.7301	0.0506
Allyl alcohol [mg.L^{-1}]	<LOQ*	<LOQ*	–	–
Methanol [mg.100cm^{-3}]	807.1 ±51.43	803 ±53.05	0.9278	0.8696
Propanol [mg.L^{-1}]	920.2 ±108.7	831.8 ±68.88	0.1902	0.0540
2-Butanol [mg.L^{-1}]	6.82 ±2.29	8.02 ±0.39	0.3781	0.1400
2-Methyl-1-propanol [mg.L^{-1}]	179.2 ±13.30	192.3 ±14.13	0.8594	0.0580
n-Butanol [mg.L^{-1}]	4.5 ±0.31	4.5 ±0.27	0.6959	1
2-Methylbutanol [mg.L^{-1}]	149.7 ±9.74	158.7 ±9.46	0.9316	0.0623
3-Methylbutanol [mg.L^{-1}]	637.8 ±40.1	643.4 ±40.5	0.9731	0.7717
Volatile matter [mg.100cm^{-3}]	480.4 ±30.08	484.4 ±29.74	0.9734	0.7799
Ethyl acetate [mg.L^{-1}]	131.1 ±9.41	138.6 ±5.12	0.0846	0.0515
Acetal [mg.L^{-1}]	37.0 ±9.23	44.6 ±5.92	0.2021	0.0522
Total hydrogen cyanide [mg.100cm^{-3}]	<LOQ*	<LOQ*	–	–
Ethyl carbamate [mg.L^{-1}]	<LOQ*	<LOQ*	–	–

Note: <LOQ* – Limit Of Quantification.

The actual alcohol content values of the plum palinka samples included in the study met the minimum value of 37.5% specified in **Regulation (EC) No 110/2008**, based on the measurements the alcohol content of the distillates were 44.08 ±0.23% and 44.0 ±0.34%, respectively.

The presence of large amounts of acetaldehyde can lead to headaches and nausea. In the samples tested by us, acetaldehyde was detected in amounts of 69.1 ±4.56 and 64.3 ±5.14 mg.L^{-1}, respectively. This is in contrast to the scientific results of **Balcerek et al. (2017)** who obtained significantly different values when comparing the two technologies.There is no limit value for this component in the regulation.

Allyl alcohol was present in the samples tested in concentrations below the limit of quantification of 2 ±0.2 mg.L^{-1}.

Methanol is converted by our bodies to formaldehyde, which is toxic and can cause blindness or even death in large quantities. In the case of the samples tested, it could be detected in amounts of 807.1 ±51.43 mg.100cm^{-3} and 803 ±53.05 mg.100 cm^{-3}, while its legal limit value is 1.200 g.hL^{-1} absolute alcohol in the case of plum distillates.

Fusel alcohols, such as propanol, butanol, and methyl butanol, are formed during fermentation, and they have a solvent odor and taste. Depending on their quantities, they may cause opacification during reconstitution. The esters of these higher alcohols with organic acids are pleasant, desirable aroma components in the distillate. Propranol and butanol are suitable for determining vintage **(Kovács et al., 2018)**, however, no difference between the technologies was found. There are no specific limit values in the regulation for the individual components. However, the limit value for the volatile matter content, which is associated with it, is set at 200 g.hL^{-1} of absolute alcohol. In the case of the plum palinka samples included in the study, the volatile matter content values were 480.4 ±30.08 and 484.4 ±29.74 mg.100cm^{-3}, respectively.

The amounts of the components ethyl acetate and acetal are not legally regulated.

Research has shown that in the case of omitted or poorly performed pitting, more hydrogen cyanide can be present in the palinka than the permissible amount, which is detrimental to health. The legal upper limit is 7 g.hl^{-1} absolute alcohol. In the case of the samples analyzed by us, the amount of hydrogen cyanide was below the limit of quantification of 1 ±0.1mg.100cm^{-3} in all cases.

Carcinogenic ethyl carbamate may form from components added as unsuitable yeast nutrients **(Deák et al., 2010; Monakhova, Kuballa and Lachenmeier, 2012)**. The regulation does not set a limit value for it, but based on the recommendation of EFSA, it is proposed that it is kept below a value of 1 mg.L^{-1}. The presence of this component could not be detected in the samples analyzed. The limit of quantification for ethyl carbamate was 0.41 ±0.041 mg.L^{-1}.

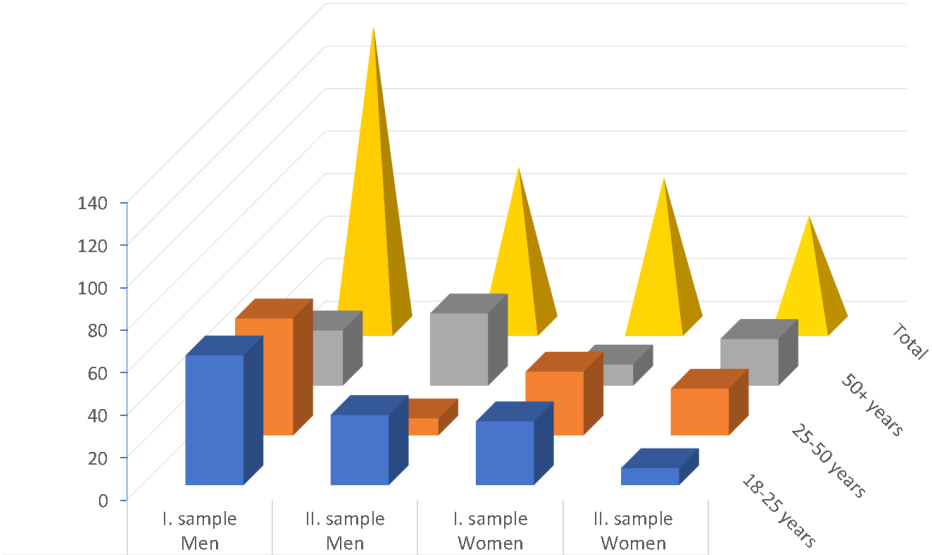
Emphasizing the importance of the testing of 15 components, the presence of propanol specifically indicates the presence of the fruit raw material, while its absence can be an indicator of counterfeiting, for example, by grain alcohol.

Legal regulations are always aimed at producing safe and healthy food. It is necessary to measure the components of the distillate and to set limit values, as exceeding them can either initiate severe irreversible processes in the human body or even cause death. Even though there are no specified limit values for fusel alcohols, however, it has been mentioned that their excessive presence gives the distillate a pungent odor and an unpleasant aftertaste. Our analyses revealed that, in the case of the samples tested by us, each batch complied with the legal requirements, i.e., the foods were safe.

However, based on previous research by **Satora et al. (2017)**, **Ismail et al. (1980)**, **Velíšek et al. (1982)**, measurements that meet the legal requirements do not include components that determine the character of plums.

Results of sensory tests

The opinions of consumers were tested by sensory analyses and this was not difficult since it was also found by **Fodor, Hlédik and Totth (2011)** that the popularity of palinka is increasing. We have 341 questionnaires completed in October 2019 at our disposal on the sensory comparison of plum palinkas produced in 2018.



	I. sample Men	II. sample Men	I. sample Women	II. sample Women
18-25 years	61	33	30	8
25-50 years	55	8	30	22
50+ years	26	34	10	22
Total	142	75	70	52

Figure 3 Results of the palinka test.

64% of respondents were male, 36% were female; in terms of age distribution, 34% were between 25 and 50 years old, 39% were between 18 and 25 years old, typically college students, while 27% were over 50 years old. The results of the questionnaire survey are shown in Figure 3.

As a result of the study it can be stated that in the comparative organoleptic study of the one-year-old products, 99.4% of the respondents (339 people) distinguished between the two palinka samples. There were only two people who found no difference between the samples. 63% of those who found a difference marked plum palinka I tastier, while 37% chose sample II. To examine that the choice depends on gender or age contingency tables (or crosstabs) were used. In terms of gender the p-value for Pearson's χ^2-test was $p = 0.141$, thus it can be stated that no difference was found between the choice pattern of the different genders. On the other hand, in the case of age groups for the p-value of Pearson's χ^2-test $p < 0.001$ holds, therefore the preference of palinka does depend on age, but this dependence is weak since the Goodman and Kruskal's λ value equals to 0.157. Although the pool of respondents cannot be considered representative, it can be stated that the sample produced using the pot-still technology (sample II) was chosen by the age group over 50, while the age group 25 – 50 found the sample prepared using the column technology tastier. According to our results, we can say as well, that young adults don't really have a clear preference of palinka with respect to the technology it was produced. A similar conclusion, that consumers are unaware of production technology processes, was reached by **Szegedyné Fricz et al. (2017)**. In Table 3 we present the corresponding contingency table. In this table, Count means the number of persons in a specific category while Expected Count shows the number of persons in the same category in the case when there would be no association between age and palinka choice. Too high difference between these values indicates that in this certain category age has an effect on palinka choice. Based on the answers given to question 1 during the sensory examinations, it can be stated that our hypothesis No. 2 can be retained, that is, the final products prepared using different distillation technologies from the same batch of raw material and the same preparation differ based on organoleptic evaluation. The answers to the second question do not explain the reason for the discrimination but confirm the difference. Naturally, another question arises as to the product made by which technology is found to be tastier by consumers, but the present case study does not examine this issue and our previous research **(Géczi, Korzenszky and Nagygyörgy, 2018)** has shown that during the relaxation and maturation of palinka, processes take place that influences the quality of palinka and the perception of being the tastier technology highly depends on the "age" of the product.

Taking into account the differences in sensory examinations, in order to compare the technologies, it would be necessary to measure the components characteristic of the product and to compare the degree of dephlegmation analyzed by **Nagygyörgy (2010)** and **Nagygyörgy (2016)**.

Table 3 Contingency table for age groups and palinka.

			palinka I	palinka II	Total
Age	18 – 25	Count	91	41	132
		Expected Count	82.5	49.5	132
	25 – 50	Count	85	30	115
		Expected Count	71.9	43.1	115
	50<	Count	36	56	92
		Expected Count	57.5	34.5	92
Total		Count	212	127	339
		Expected Count	212	127	339

CONCLUSION

Based on the measurement results it can be stated that there is no significant difference in the legally specified and tested 15 content values between the two plum distillates produced from the mash prepared from the same raw material and the same preparation technology but using two various distillation technologies. No differences could be detected between the final products prepared using the pot-still technology (PSDD) and the rectification column technology (RCDS) with accredited instrumental analyses, so our No.1 hypothesis was rejected.

However, in the case of organoleptic analyses, participants in the study made a clear distinction between the samples tested. In each case, they found one of the samples tastier than the other. It can be stated that the final products made using the two different distillation technologies can be clearly distinguished from each other based on organoleptic tests, but the analytical tests could not support this result. To support this, we consider it necessary to perform analytical determination of further components specific to plums. Legally prescribed qualification parameters generally do not include components specific to distillates made from the individual fruits, such as benzaldehyde, methyl cinnamate, and y-decalactone in the case of plums. Regardless, our hypothesis No. 2 was accepted. Consequently, we are developing new measurement and evaluation methods to investigate the cause of the difference.

As a result of our tests, it can be stated that the analytical parameters of the legal requirements are not suitable for the comparison of palinkas produced using various technologies.

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THE USE OF CONSUMER NEUROSCIENCE IN AROMA MARKETING OF A SERVICE COMPANY

Jabuk Berčík, Anna Mravcová, Jana Gálová, Martin Mikláš

ABSTRACT

In today's highly competitive era, it is becoming increasingly difficult for businesses to attract and keep customers. Modern marketing strategies focused on examining customer behavior, and possibilities of the business sector in influencing them are becoming increasingly beneficial. Consumer neuroscience has great potential here, as it reveals internal consumer preferences by using innovative techniques. Human senses play an important role in affecting these preferences, and smell has the greatest potential to influence customers subconsciously, and thus support the sale of products, keep customers in the store longer, or build a brand. Aroma marketing is the continuously developing field of consumer neuroscience. The paper aims to examine the use of consumer neuroscience in aroma marketing of a service company. After theoretical analysis, we carried out practical research aimed at examining the impact of aroma marketing in a selected service company – Sport Café in Nitra – to increase the sales of a particular product, and its real impact on the economic indicators of this company through the use of consumer neuroscience tools and questionnaire survey. In aromatization, choosing the right aroma is of the utmost importance, therefore in our research, this selection was done in the Laboratory of Consumer Studies at the Faculty of Economics and Management, SUA in Nitra. The research was carried out through conscious and unconscious testing of selected aromas by a randomly chosen sample of customers. Conscious testing included using a questionnaire method and unconscious testing was done using a face-reading device that provides an objective assessment of emotions based on facial expressions. The choice of aroma was followed by testing in real conditions, by diffusing the aroma in the café. Based on the collected data, we confirmed the positive impact of aromatization on coffee sales. In conclusion, we present some recommendations for service companies, focused mainly on cafés, with emphasis on the importance of aromatization for attracting and keeping customers and improving profitability.

Keywords: consumer neuroscience; aroma marketing; service company; aroma; Sport Café

INTRODUCTION

The current era is characterized by intensifying competition of business environments and entities. It is becoming increasingly difficult to attract as well as keep customers. It is why new strategies and approaches are increasingly spreading in the marketing environment, which focuses on examining consumer behavior and the possibilities of influencing it positively by enterprises. To understand what guides consumers' steps and behavior during shopping in a world of advertisement overload, traditional marketing tools seem to be still less effective, because the majority of people´s thoughts are in the subconscious mind and consumers mostly do not choose products rationally. The trend nowadays is the communication simultaneously oriented on several human senses, which is represented by the modern field of consumer neuroscience, or neuromarketing, which is using neuroscience to reveal subconscious consumer decision-making processes **(Berčík et al., 2018; NMSBA, n. d.; Genco, Pohlmann and Steidl, 2013)**.

Consumer neuroscience refers to the measurement of physiological and neural signals to gain insight into the customers' motivations, preferences, and decisions, which can help improve creative advertising, product development, pricing, and other marketing areas **(HBR, 2020)**. By this, marketers learn why consumers make the decisions they do, and what parts of the brain are motivating them to do so.

Neuromarketing is focusing on understanding the thoughts and behavior of consumers to "transfer insights from neurology to research in consumer behavior by applying neuroscientific methods to marketing relevant problems" **(Miljković and Alčaković, 2010)**. Therefore, it is an interdisciplinary field that combines aspects of neuroscience, psychology, and marketing. It represents a marketing strategy that is connected to the subconscious, emotional aspect of the customer and it then wants to create a strong bond with the customer and the product **(Karmarkar, 2011)**. The goal is to study how the brain is physiologically affected by advertising and marketing

strategies, and how to improve their effectiveness. Therefore, "neuromarketing studies are focusing on obtaining objective information about the inner workings of the brains of consumers" **(Miljković and Alčaković, 2010)**.

According to **Labská, Tajtáková and Foret (2009)**, the general goal of marketing communication is to influence buyer behavior Every business entity wants to present its products and services in a way that strengthens customers' interest in buying them.

We agree with **Kulynych (2002)** that neuromarketing focuses on measuring the proper functioning of marketing in practice, and it is based on the realistic understanding of how the customer's brain works. So, neuromarketing can be understood as a new era of marketing that seeks the direct impact of marketing incentives on customer and consumer responses at the time of purchasing decision-making **(Ferguson, 2009)**. Neuromarketing, as a scientific discipline using advanced methods, brings a new perspective on consumer shopping behavior. This significantly affects the subsequent creation of marketing strategies and integrated marketing communication in all its areas. Looking into the minds of customers, finding out and researching what they react or do not react to, is the subject of many discussions **(Púchovský and Kohoutová, 2015)**. Predicting customer behavior is of great importance to marketers and the business environment as then they can offer the customer what he wants.

The importance of neuromarketing potential is underlined by the finding that about 95% of thoughts occurred in the subconscious mind cannot be measured by traditional research methods **(Marketing-Schools, 2012)**, whereas these thoughts greatly influence emotions, and decision-making thus begins before all the provided information is obtained **(Genco, Pohlmann and Steidl, 2013)**. Neuromarketing using various techniques, such as those used in medicine and psychology, tries to identify consumer preferences more accurately. It is a more detailed examination of the economic behavior of consumers. Besides, it is also used in the development, creation, and placement of products on the market **(Berčík et al, 2016)**. Neuromarketing uses brain imaging, scanning, and other brain activity measurement technologies to measure the response to a specific product, packaging, advertisement, etc. **(Neuroscience marketing, 2019)**. Phan states that it uses these medical technologies to determine consumer reactions to those particular marketing elements **(Phan, 2010)**.

We recognize several technologies here. **Berčík et al. (2016)** divide research tools and neuromarketing techniques into two basic categories. Each approach captures a different type of signal:

Neuroimaging measurements – devices measuring brain responses under the influence of marketing stimuli. The two most important for scanning the brain are *fMRI* (functional magnetic resonance imaging) and *EEG* (electroencephalogram). However, they are expensive, and fMRI is also too large. FMRI uses strong magnetic fields to monitor changes in blood flow across the brain. It examines brain functions through a three-dimensional display of the brain. EEG reads brain-cell activity using sensors placed on the subject's scalp **(HBR, 2020)**. When a stimulus is applied, the neurons create electric currents that create brain waves, and the device displays them as curves. It measures attention, engagement/boredom, excitement, cognition, memory coding, or emotional valence. These tools are used, for example, to test new products, advertisements, product placement on shelves and identify customer needs **(Púchovský and Kohoutová, 2015)**.

Biometric measurements – tools measuring the physiological proxies for brain activity. They tend to be more affordable and easier to use. These include:
Eye tracker can measure attention (via the eyes' fixation points) and arousal (via pupil dilation). It allows us to examine consumer behavior by the point of gaze, how long it lasts, and also the motion of the eye. It measures the intensity and frequency of gaze.
Face reader is about facial-expression coding as it reads muscles in the face. It measures emotional responses through the face and represents an automatized facial expression analysis software that provides an objective assessment of emotions. It is fast, flexible, objective, and easy to use.
Heart rate variability (HRV) is a physiological marker of how we experience and regulate our emotions. It represents the number of seconds that pass between one heartbeat and the next, which is called the interbeat interval **(Aldo, 2014)**.
Galvanic skin response (GSR) analyses changes in the sweat gland activity of the skin when the vegetative nervous system is activated. It measures emotional arousal and can distinguish between real excitement and noise, but it is not able to distinguish between positive excitement and stress **(Púchovský and Kohoutová, 2015; HBR, 2020)**.

Aroma marketing

In this field, sensory marketing has an increasing role. It involves communication with consumers through sight, hearing, smell, touch, and taste to influence perceptions, judgments, and behaviors of consumers through their senses to create a pleasant environment, so that the customer increases the purchase time at a particular place **(Krishna, 2012; Jiménez-Marín, Bellido-Pérez and López-Cortés, 2019; Bilek, Vietoris and Ilko, 2016; Tauferova et al. 2015)**.

We can say that the nose is one of the most sensitive and emotional senses with a great ability to associate certain aromas with specific situations. Neuromarketing related studies affirm that 75% of our emotions experienced during the day are related to aromas, and humans can distinguish around 10,000 different aromas **(Bell, 2006; Minsky, Fahey and Fabrigas, 2018; Jiménez-Marín, 2016; Erenkol, 2015)**. They also strongly influence buying behavior. As several types of research indicate, while humans are only able to remember 50% of the things they have seen after three months, in the case of aromas it is up to 65% after one year **(Mukherjee, 2015)**. Thus, aroma marketing has big potential in marketing and can be utilized in the business environment and services.

Aroma marketing has still not been fully discovered yet, however, it can play an enormous role in supporting shopping processes and human behavior, since smell has an advantage over other senses, because it can stimulate human emotions immediately. Using aromas, marketers can create a connection with customers at a deeper emotional level and provide them an unforgettable experience.

The smell is one of our most primal and deeply rooted senses and it functions as our chemical alert system. With all other senses, the person thinks before he responds, but with aroma, the brain responds before humans think **(Vlahos, 2007; Aroma Marketing, 2020)**. The smell can create direct reactions to marketing stimuli as it is most closely related to emotional reactions. The smell is the only sense directly connected with the limbic system, which controls memory and emotions. Humans react emotionally to some aroma even before they can identify it. This has a subconscious effect on their behavior and consequently on a company. Customers affected by the nice smell in the store stay 44% time longer than they would without the pleasant aroma **(Conick, 2017)** and this significantly increases the impact of aroma marketing on the store's profit.

However, smell perception varies, and it involves many factors, including individual preferences. Therefore, the most important is to find those aromas that will attract as many potential consumers as possible **(Virkkunen, 2015)**. By this, aroma marketing becomes an essential part of marketing communication **(Sikela, 2015)**. According to the studies and above-mentioned knowledge, our smell is the strongest sense about memory, finding that we are 100 times more likely to remember something that we smell in comparison with something that we see, hear, or touch **(Vlahos, 2007)**. All senses evoke memories, but smell evokes more emotional ones **(Nadányiová, 2017)**. The positive results about the use of aroma in a business environment suggest "that customer satisfaction can be increased through thoughtful manipulation of ambient stimuli" **(Bradford and Desrochers, 2010)**.

Aroma marketing is more than just diffusing a pleasant aroma in a space. It is the art of combining the brand – a company identity with aroma, and thus highlighting the brand and ensuring that it is differentiated from the competition. It is not only about the induction of impulsive consumer shopping behavior, but also about the support of the company's image, as well as identification or recognizability of the communicated product, service, and company **(Füziková and Madleňák, 2017)**. The right application of aroma contributes in particular to a positive evaluation of the company by customers, to a better perception of the company's products and services, and to the fact that customers subconsciously tend to return to the companies that use aroma marketing **(ScentAir, 2020)**. Businesses thus often have their aroma – the so-called aroma logo – mixed following the overall image of the brand and its environment. When shopping, the customer does not think about the aroma, but if one comes to the store and smells it, they automatically remember the brand without seeing its logo. Aroma branding aims to create an immediate association with the brand when smelling it so that it expands brand recognition which can also attract new customers, but especially strengthen the brand image, increase customer satisfaction and sales, create a brand association, build a relationship with the brand, induce a positive perception of products, services, and staff, as well as extend the customer's time spent in the store and increase their spending patterns **(Spectrio, 2020; Air/Aroma, 2020; Berčík et al., 2016; Paluchová, Berčík and Neomániová, 2016; Cartwright, 2014)**.

Applying the right aroma also helps to improve the mood not only among customers but also among employees, and tends to make customers more generous **(Jurášková and Horňák, 2012)**. We agree that special emphasis should be placed on selecting the appropriate aroma because selecting one that does not suit the product/service or if its intensity is adjusted incorrectly, may have the opposite effect **(Bradford and Desrochers, 2010)**. Therefore, an important task for market specialists and store owners is to find the right aroma and the right intensity, and it is necessary to examine it before its implementation, to avoid these problems and mistakes **(Berčík et al., 2017)**.

Scientific hypothesis

To examine the use of neuroscience in aroma marketing of a selected service company, it is essential to examine the importance and potential of the use of neuromarketing in aroma marketing of restaurants/cafés. As we mentioned above, the use of aroma can significantly positively influence consumer behavior, increase the productivity of employees and, last but not least, create positive and long-term emotions associated with the brand. When entering any sales space, including a café, the first impression is very important. It decides on whether the customer will feel pleasant, thus the use of aromatization with a suitably selected scent is even more important.

The service company selected for our research was the Sport Café in Nitra, where we tested aroma marketing and its influence on the marketability of coffee as our selected product, and ultimately its effect on the economic indicators of the company using consumer neuroscience tools. For this purpose, we also set an assumption that when using aromatization, the sales of coffee are higher than without it, which we verified with our research.

MATERIAL AND METHODOLOGY

The research, originating from theoretical analysis, was focused on a selected service company and on examining aroma marketing through the use of consumer neuroscience tools as well as a questionnaire survey. The research was carried out in the laboratory and also in real conditions, at the Sport Café located in Nitra in the City Hall building. After testing in a laboratory, and the aroma was chosen for testing in real conditions, through which we verified the defined assumption that aromatization used in a selected café increases coffee sales.

For the research, the sales of coffee types were monitored in the selected company in February (between February 1, 2020, and February 14, 2020): in the morning, in the afternoon, and total for the whole day.

During this period, a target sample of 8 respondents visiting the café was selected. The test was carried out on this sample, aiming at deciding which aromas are suitable for the café, to select the most suitable one for the subsequent aromatization of the café space. The test was performed in the Laboratory of Consumer Studies at the Faculty of Economics and Management SUA in Nitra and the sample of respondents involved 4 men and 4 women.

During the testing in laboratory conditions, in addition to traditional feedback, unconscious feedback was also obtained by monitoring visible changes in mimic muscles and dilation of eye pupils using facial biometrics. We were

interested in the conscious and unconscious preferences of the respondents and their emotional responses. Therefore, laboratory testing was performed in two ways – conscious perceptions testing (through a questionnaire) and unconscious perceptions testing (through face reader):

- Conscious and unconscious testing of blind samples was carried out simultaneously. 7 types of coffee aromas were tested – 1. Coffee Pure, 2. Coffee and Cake, 3. Cappuccino Cocoa, 4. Sugared Almonds, 5. Cappuccino, 6. Cappuccino Amaretto, 7. Coffee House. After smelling the sample for at least 10 seconds, the respondents evaluated their conscious preference, which they expressed in an electronic questionnaire form.
- At the same time, their emotional feedback was monitored through a facial biometric tool – it was the testing of unconscious (implicit) reactions using a face-reading device. Respondents sat behind the table and while testing each aroma, they were looking into the camera that captured and evaluated their unconscious reactions using automated software, based on their facial expressions (see Figure 1).

Subsequently, we carried out the testing in real conditions, when based on the results obtained from laboratory testing, we aromatized the space of the café for 2 weeks, from February 29, 2020, to March 13, 2020. Based on laboratory tests, the Coffee House aroma was used for aromatization. We placed the aroma streamer device in the café, away from the door and the ventilation. Using the Reima mobile app, we connected to the device via Bluetooth and set the aroma release interval and its intensity.

After testing, we processed the results of coffee sales during aromatization and compared them with the research period before aromatization. After evaluating the results, we formulated conclusions and recommendations for practice.

Emotional feedback was monitored using the somatic biometric method Facereader 7 from the Dutch company Noldus, which identifies the emotional feedback (valence, excitement) of the respondents with maximum accuracy based on observable changes in mimic muscles and recognizes basic micro-emotions (happy, sad, angry, disgusted, surprised, neutral).

During the test, it was also necessary to check the air quality in the room, which was monitored by the Air Quality data logger device, which enables the real-time display of values of basic air quality parameters such as CO_2, temperature, humidity, volatile substances VOC, and dust particles.

Statistical analysis

The data obtained from the measurements were synchronized and correlated in Observer XT 10 by Noldus. This program allows to synchronize structured and unstructured data from different devices and at the same time to create their variables during the implementation of experiments. Processing was carried out in the Matlab R2019a and Microsoft Excel 2010 program environments.

Descriptive and inductive statistic methods were used to process the primary data. As part of inductive statistics, we used the Mann-Whitney U test (one-sided) to verify our hypothesis. It is a test of the agreement of two mean values for independent files (parametric equivalent).

RESULTS AND DISCUSSION

Aromas can create lasting memories, eliminate stress, and support the creation of emotions. Thanks to an appropriately chosen aroma in the store, the seller can influence the behavior of customers, who then can evaluate the company more positively, tend to subconsciously return to these spaces, perceive the higher quality of products and spend more time there. Hence, we focused our research on verifying the assumption that the aroma implemented within the store will increase coffee sales. Our research about the use of consumer neuroscience in aroma marketing of a service company was carried out first in the laboratory and subsequently in real conditions.

In the laboratory, we tested the conscious and unconscious reactions of the eight chosen respondents (who are visitors of the café) to the 7 selected aromas associated with coffee. The first part of the testing consisted of a conscious response to the tested aromas while completing a questionnaire. The results here showed that respondents liked Aroma 7 (Coffee House) the most (see Figure 2).

In the second part of the laboratory testing, the unconscious reactions of the respondents to the choice of aromas were examined and recorded using the Facereader device, where we found out that our respondents perceived Aroma 5 (Cappuccino), the most positively. The most negatively perceived aroma here was Aroma 7 (Coffee House), as seen in Figure 3.

During the test, we were checking the air quality in the room using the Air Quality data logger device. We focused primarily on CO_2 concentration, as it can affect human preferences the fastest. Attributes should be up to the CO_2 level of 700ppm (see Figure 4).

Based on the results of laboratory testing, we placed an aromatizing unit directly in the selected café. However, the results from laboratory testing were contradictory. The Coffee House aroma was perceived most positively during conscious testing, but it was also evaluated most negatively by unconscious testing. There can be several reasons for this. One option is that the aroma was too intense, which had an immediate strong effect on the olfactory organ and the facial expression could therefore seem negative. But ultimately, it had a positive effect on the smell. It is not always possible to obtain an explicit result when examining unconscious feedback, as the process may be affected and distorted by various side factors. In real conditions, we, therefore, decided to deploy and test the Coffee House aroma that was most positively evaluated in conscious testing and this was implemented in the Sport Café. For the aromatization, we used the aroma streamer unit placed in the company. Using Bluetooth, the aroma release interval was set on the device, which was daily from 11:00 to 20:00, and the intensity was set at level 7.

We then compared the coffee sales before the aromatization with the sales of coffee during the aromatization. The results are presented in Table 1 and Table 2, which contain the date of recording, the number of coffees sold in the morning and the afternoon, and then the total sum of coffees sold. The types of sold coffees were: 1. Turkish coffee; 2. Espresso, 3. Decaffeinated coffee; 4. Viennese coffee; 5. Latte Macchiato; 6. Cappuccino; 7. Iced coffee.

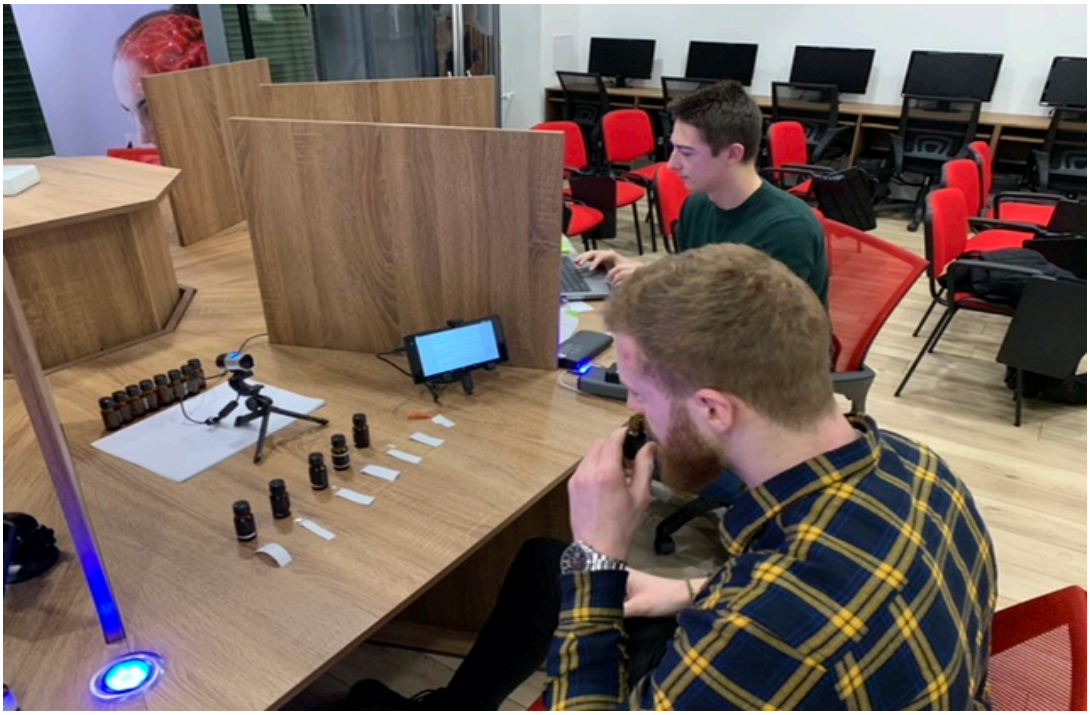

Figure 1 Testing in laboratory conditions. Note: Source: Own evaluation, 2020.

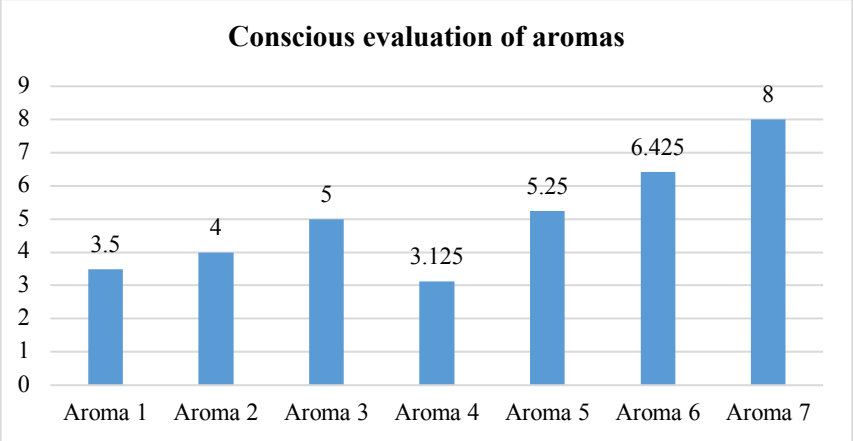

Figure 2 Conscious evaluation of aromas. Note: Source: Own evaluation, 2020.

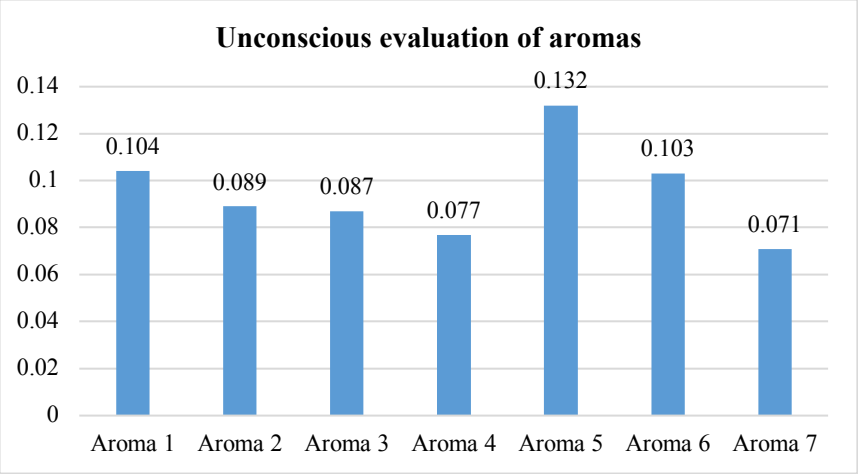

Figure 3 Unconscious (implicit) evaluation of aromas. Note: Source: Own evaluation, 2020.

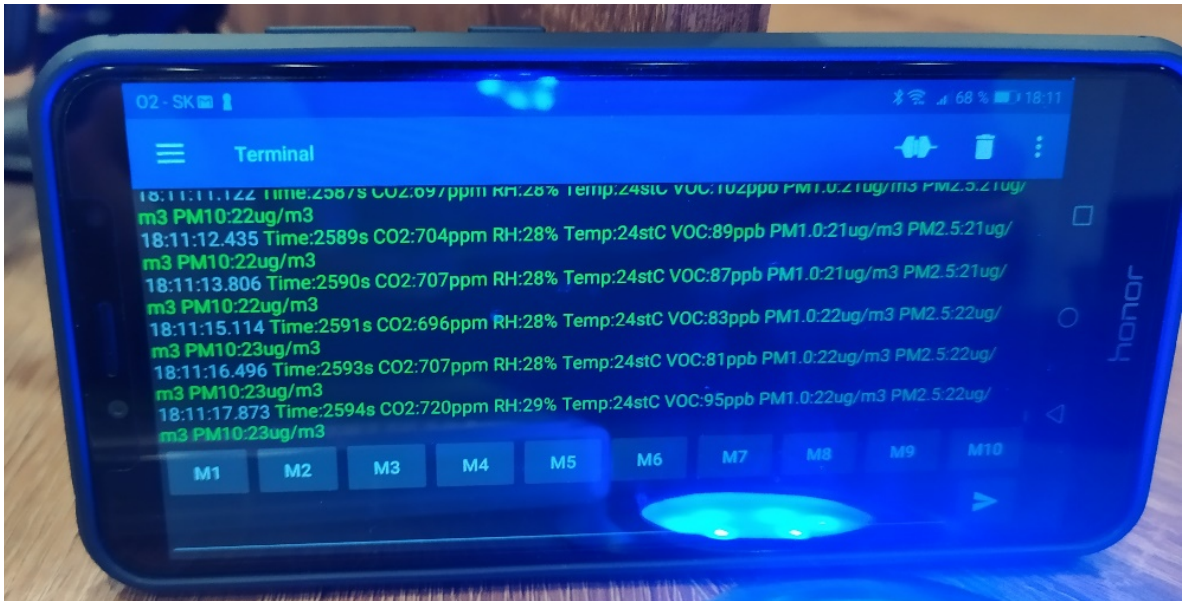

Figure 4 Air quality measurement. Note: Source: Own evaluation, 2020.

Table 1 Number of coffees sold in the period without aromatization.

Date	Morning							Afternoon							SUM
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	
1.2. SAT	0	4	2	1	3	6	0	0	4	1	0	2	4	0	27
2.2. SUN	1	3	0	2	3	4	0	0	2	1	0	1	3	0	20
3.2. MON	0	2	0	1	0	3	0	0	2	0	0	0	2	0	10
4.2. TUE	1	2	0	2	1	3	0	1	1	0	0	0	1	0	12
5.2. WED	0	3	0	2	1	4	0	0	1	0	1	0	2	0	14
6.2. THU	0	4	0	2	0	4	0	0	1	0	0	1	3	0	15
7.2. FRI	0	3	0	1	1	4	0	1	1	0	1	1	2	0	15
8.2. SAT	0	5	0	2	3	6	0	0	4	0	2	2	6	0	30
9.2. SUN	0	5	0	1	1	5	1	0	3	0	2	1	3	0	22
10.2. MON	0	3	0	1	1	4	0	0	1	0	0	0	2	0	12
11.2. TUE	0	2	0	2	2	2	0	0	2	0	0	0	2	0	12
12.2. WED	2	3	0	0	1	3	0	0	2	1	0	0	2	0	14
13.2. THU	2	3	1	0	0	2	0	0	2	1	0	1	2	1	15
14.2. FRI	2	5	1	0	0	6	0	0	2	0	0	1	3	2	22
SUM	8	47	4	17	17	56	1	2	28	4	6	10	37	3	240

Note: Source: Own evaluation, 2020.

Table 2 Number of coffees sold in the period with aromatization.

Date	Morning							Afternoon							SUM
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	
29.2. SAT	2	5	2	1	3	7	0	2	5	1	1	2	6	0	37
1.3. SUN	1	3	0	2	4	5	0	0	3	2	1	1	5	0	27
2.3. MON	1	3	0	1	0	5	0	1	2	0	0	0	2	0	15
3.3. TUE	2	3	0	2	1	4	0	1	2	0	0	0	2	0	17
4.3. WED	1	3	0	2	1	5	0	1	2	0	1	0	2	0	18
5.3. THU	0	4	0	2	2	4	0	1	3	0	0	1	5	0	22
6.3. FRI	2	4	0	1	1	6	0	2	3	0	1	1	4	0	25
7.3. SAT	2	6	0	3	3	6	0	1	4	0	2	2	6	0	35
8.3. SUN	0	5	0	2	2	5	1	1	3	0	2	2	3	0	26
9.3. MON	2	3	0	1	1	4	0	0	1	0	0	0	3	0	15
10.3. TUE	0	3	0	2	2	3	0	0	2	0	0	0	2	0	14
11.3. WED	2	3	0	0	1	5	0	0	2	1	0	0	2	0	16
12.3. THU	2	3	1	0	0	4	0	0	2	1	0	1	3	1	18
13.3. FRI	2	5	1	2	0	6	0	0	2	0	0	1	4	2	25
SUM	19	53	4	21	21	69	1	10	36	5	8	11	49	3	310

Note: Source: Own evaluation, 2020.

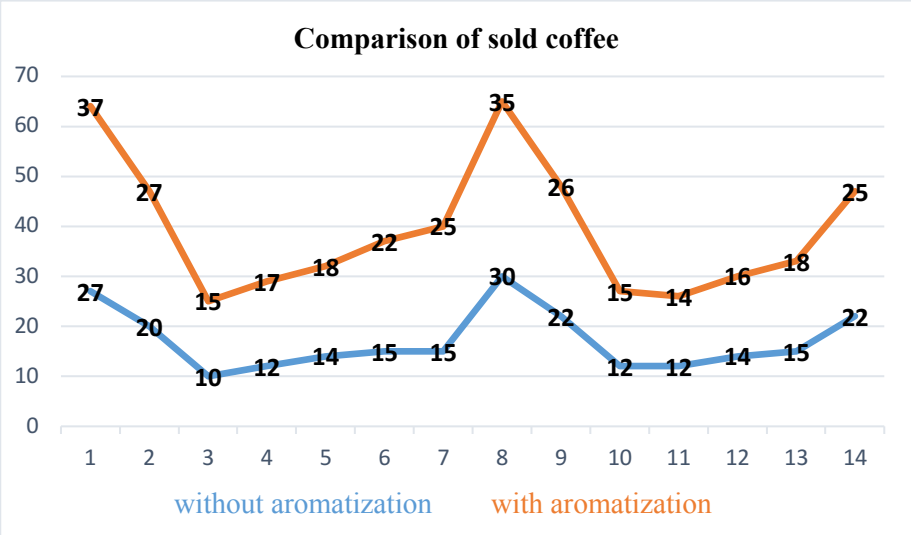

Figure 5 Comparison of sold coffee. Note: Source: Own evaluation, 2020.

In the 2 weeks before aromatization (February 1, 2020 – February 14, 2020), 240 coffees were sold in the café, from these 150 in the morning and 90 in the afternoon. Cappuccino and Espresso were sold the most, while iced coffee was sold the least – the reason might have been the season – as well as Decaffeinated coffee since it is a specific product, which customers do not seek very much in Slovakia (see Table 1).

During the 2 weeks of aromatization of the Sport Café with the Coffee House aroma (from February 29, 2020, to March 13, 2020), we recorded an increase in coffee sales by approximately 30%. Sales increased by 70 coffees, with the total number of coffees sold in this period being 310. Most coffees (188) were sold in the morning and 122 in the afternoon. The cappuccino was sold the most, then Espresso, while sales of Turkish coffee increased too (see Table 2). For a better presentation and comparison of sales, we summarized the results in Figure 5.

During our research, we successfully verified the stated assumption using a Mann-Whitney test – one-tailed (p-value of 0.015 is less than alpha 0.05, we reject A0 and accept A1), so when using aromatization, the sales of coffee were higher than without it.

Examining consumer behavior is of great interest in many research and studies in various fields, focused mostly on the goal to make the products more attractive to the consumers (see, for example, **Karmarkar, 2011; Miljković and Alčaković, 2010; Kozelová et al., 2013; Kozelová et al., 2011)**. Many studies and research are focused on food and drinks where a wide range of attributes are being examined **(Vietoris et al., 2016; Zinina et al., 2019; Zajác et al., 2015)**.

There are also many studies focusing on the effect of aromas on consumer behavior (see, for example, **Conick, 2017; Virkkunen, 2015; Bradford and Desrochers, 2010; Berčík et al., 2017**) and several are aimed at service companies that have similar results confirming the increase of product sales after using aromatization. There was, for example, a study carried out in a restaurant where the lavender aroma was diffused and compared to a no-aroma controlled condition. Results showed that lavender caused customers to stay longer and increased the amount of purchasing since it seemed to relax people. When relaxing, the customers ordered additional items and thus increased the amount spent **(Guéguen and Petr, 2006)**. Another study was done, for example, in the Brooklyn food market NetCost which aromatized delicious smells through their aisles. After coordinating the scents with store sections, such as putting rosemary close to freshly baked bread, they saw at least a 7% sales increase. Since smell extends appetite past normal boundaries, customers were more likely to spend money **(Pulido, 2019)**. Also, according to Swedish researchers, good smells can heighten the will to buy, for example, coffee or sweet rolls. In their research in front of the Stockholm café using Eyetracker (see also, **Wästlund, et al., 2010)**, those participants who were exposed to the smell of chocolate chip cookies were 40% more likely to buy something in the café compared to the participants who were not exposed to the aroma. According to these researchers, café-related scents increased the intention to buy something **(Shams, 2013)**. Participants who were exposed to the aroma were more willing to buy also other café products **(Shams, 2013; Stranden, 2016)**.

In summary, we can conclude that our study has some limitations. Only one café was examined, and the sample of customers tested was small, and there were contradictory results from laboratory testing. However, given the theoretical research that showed the effect of aromas on customers' purchasing behavior, according to the results of this study and other similar researches, we can confirm that it is beneficial for cafés and other service companies to aromatize their spaces to influence their customers and increase their expenditures. We agree with the statement, that profit is the driving factor behind marketing decisions for cafés, restaurants as well as other businesses, and manipulating aromas can be an attractive financial move as the right smell can encourage consumers to buy more than they usually would **(Pulido, 2019)**.

CONCLUSION

Based on our research, we can state that the use of aroma marketing and the implementation of aromatization in a business space has great potential in the context of a positive impact on economic indicators of the company, including services companies. At the same time, the use of various consumer neuroscience tools is important for the deployment and application of aroma marketing in such companies, which can significantly help in selecting a suitable aroma, it's diffusing and setting the intensity of aromatization. Since smell is associated to a large extent with the reactions of the subconsciousness, it may differ from the conscious one, without being perceived. Therefore, it is necessary to supplement it with testing conscious reactions, to carry out several tests, and use different methods and tools for the correct implementation of aroma marketing and aromatization, to select the most suitable aroma and to achieve the greatest possible positive impact on customers.

Based on the results of our research on the use of consumer neuroscience in aroma marketing of a service company and on examining the coffee sales during the application of aroma marketing in the selected café, we conclude by formulating several proposals and recommendations that we believe could help positively increase economic indicators.

We recommend implementing aroma marketing permanently, as the aromatization of the space has contributed to increased coffee sales, confirmed by sales numbers and the statistical test. It is also a space for creating aroma branding, an opportunity to change the customer perception of the café, attract new and retain old customers using a pleasant aroma. Deploying a coffee aroma in a selected company significantly improved the economic indicators when coffee sales increased by approximately 30%. Although the costs of aromatization are not negligible, even after their implementation, the company's sales would be higher than without aromatization, based on the calculated expected financial inputs. Revenues from sold coffee in the examined period without aromatization were calculated as 480 euros per 2 weeks, while in the period of aromatization it was 620 euros per 2 weeks (according to the set prices of the company´s menu). The difference, in this case, is 140 euros more per 2 weeks. The monthly cost of an aroma is approximately 60 euros and the rent of the aromatizing unit is 20 euros, meaning the business would still be more profitable. Certainly, this can also mean that

sales will gradually increase even more in the longer period, and aromatization will attract more new customers, so we assume that economic indicators would change even more positively. Therefore, we recommend the selected company, as well as service companies in general, to aromatize their spaces, but it is necessary to expose the choice of aroma to proper in-depth testing.

We also propose including seasonal products in the menu, such as Pumpkin Latté in autumn, since adapting aromatization to this can also help to improve the economic indicators of the company. This could attract more customers and bring the company something new that differentiates it from at least part of the competition. The cost of preparing 1 Pumpkin Latte would be 1 EUR since it only requires pumpkin puree, spices, and one cup of espresso. The company could sell it for 2.50 EUR, as it would be a new and seasonal product. The price is set based on the competition that sells similar types of special coffees in Nitra.

Coffee sales could also be increased by including cakes into the menu, e.g. a cheesecake which would revive the company's range. It would be advisable to start with one or two cakes first (to offer some options due to food allergies, etc.), to find out if customers are interested in this type of product. The main purpose of selling cakes would be to increase sales of coffee (cross-selling). The costs involved are related to procuring the cake(s) and transport. Cake from Babičkina cukráreň company in Nitra with regular intake would cost 22 euros for 12 pieces, which is 1.80 euros per piece. Sport Café could sell a piece for 2.5 euros, which would make a profit of 0.70 cents per piece. The types of cakes could be selected based on a specific season and the final impression would be enhanced by the seasonally set aromatization.

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PREFERENCE MAPPING OF SLOVAK CHEESE

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ABSTRACT

The production of steamed cheese has a long tradition in Slovakia. Some of these cheeses have even received the PGI designation, which is a designation granted to products with specific geographical characteristics (Slovenská parenica, Zázrivské vojky, Zázrivský korbáčik, and Oravský korbáčik). In our study, eight samples of various unsmoked steamed cheese from small dairy farms (samples D, F, G, and H) and medium-sized farms (A, B, C, and E) were evaluated. Our work aimed to determine whether there are significant differences in sensory characteristics between samples from small and medium-sized dairy farms and whether there are differences in the preferences between these samples for consumers. Samples were evaluated by sensory analysis, where the assessors evaluated the characteristics of color, odor, texture, flavor, and overall appearance on a nine-point hedonic scale. Differences at a statistically significant level in the attributes of odor, texture, flavor, and overall appearance were confirmed (p <0.05) between samples from small and medium dairy farms, no statistically significant difference was proved in the attribute color (p >0.05). The results were processed using PCA, whereas can be seen from the graphic representation the carriers of all evaluated attributes were samples from medium-sized farms (except for sample D, which belonged to the first group of samples together with samples from medium-sized farms). We constructed a preferential map by combining internal and external mapping, while the internal data were formed by data obtained from assessors using sensory analysis and the external data came from an online questionnaire. Using the preferential mapping technique, we found out that samples from medium-sized dairy farms were classified as the most preferred samples which correspond with the results of sensory analysis.

Keywords: steamed cheese; unsmoked; preference mapping; sensory analysis

INTRODUCTION

Cheese production is one of the traditional ways of food preservation. Preservation of fats and proteins as the most important components of milk in the form of cheese uses two principles of food preservation: lactic acid fermentation and water activity reduction by removing water and adding NaCl **(Fox et al., 2004)**. There are more than 1000 types of cheese in the world, which differ from each other in sensory and chemical characteristics **(Montel et al., 2014)**.

Production of steamed cheese has a long tradition not only in Slovakia but also in eastern Europe and Balkan countries. This type of cheese is according to **Čuboň et al. (2015)** called Pasta filata. Traditional Slovak steamed cheese can be found in many different shapes and sizes, smoked or unsmoked, made from pasteurized or unpasteurized milk, bovine, ovine or mixed origin, etc. Characteristic shapes are strings (Zázrivské vojky), little whips (Zázrivský korbáčik, Oravský korbáčik), and most traditional „S“ shape (Slovenská parenica) **(Tasteatlas, 2020)**. Zázrivské vojky **(Commission Regulation (EU) no 963/2014)**, Zázrivský korbáčik **(Commission Regulation (EU) no 238/2011)**, Oravský korbáčik **(Commission Regulation (EU) no 243/2011)**, and Slovenská parenica **(Commission Regulation (EC) no 656/2008)** are included in the PGI and PDO list of the European Union. According to **Council Regulation (EC), no 510/2006** traditional Slovak parenica cheese is described as steamed cheese that can be unsmoked or smoked, traditionally manufactured and shaped in „S“ shape.

In recent years there have been several food scandals that decreased consumers´ trust in food safety **(Kozelová et al., 2013)**. Consumers´ demands for food with high-quality are still growing **(Rana and Paul, 2017)**. For the last few years, there has been increasing attention towards food quality and food safety and also growing demand for „natural“ products (mostly those with PGI, PDO, and TSG designation) **(Todaro et al., 2017)**.

From the point of view of human nutrition, as well as from the economic point of view, the dairy industry belongs to the main branches of the food industry in the Slovak Republic. In line with the global trend, the consumption of cheese and curds has increased, with consumers preferring natural cheese over processed

cheese. The largest volume of the total value of foreign trade in the dairy industry consists of cheese and cottage cheese. The export of Slovak cheese is mainly focused on the EU-28 market **(Gálik, 2019)**.

Consumption of milk and dairy products without butter in 2018 was 171.1 kg, which was less by 3.5 kg (2.0%) compared to the year 2017. The decrease in consumption was mainly reflected in sour milk products by 0.6 kg (3.4%) and cheese and curds together by 0.2 kg (1.5%). Because milk and dairy products are the main sources of calcium and protein, their low consumption is nutritionally unfavorable. In 2017, according to available data from Central European countries, for example, the Czech Republic (239.3 L) and Poland (218 L) had a high consumption of dairy milk in the value of butter-free milk per capita. In the Slovak Republic, the consumption of milk was 169.5 L per capita. Hungary had a comparable value of consumption as in Slovakia in 2017 with 160.7 L per capita. The total consumption of cheese and curd in 2018 was 13.3 kg per capita in the Slovak Republic, in 2017 it was 13.5 kg **(ŠÚSR, 2019)**.

Preference mapping is a methodology that helps us to identify the sensory attributes which are the main drivers of liking and the most preferred products for different consumer groups. This technique uses multivariate tools to create bidimensional plots (maps) that link product characteristics and consumer preferences **(Berget et al., 2020)**. Preference mapping has become a standard tool not only in sensory studies of food but also in other product categories **(Mattila, 2001; Zacharov and Koivuniemi, 2001)**. For performing preference mapping liking/preference data obtained from consumers and a descriptive sensory profile for the same products is needed. Firstly, PCA (Principal Component Analysis) is used on the independent data, then the dimension reduction techniques are applied depending on the data. There are conceptually two different ways of performing preference mapping – internal and external. In internal preference mapping, the sensory data is modeled from the consumer data and in external preference mapping the order is switched, so consumer preferences are predicted from sensory data **(Berget et al., 2020)**. Both internal and external approaches have their pros and cons **(Næs, Varela, and Berget, 2018)**.

Our work aimed to find out the preferences of consumers of unsmoked steamed cheese from small and medium-sized producers and confirmation that there are differences in sensory characteristics between cheese from medium-sized and small farms.

Scientific hypothesis

Hypothesis 1: There exist significant differences in sensory attributes between unsmoked steamed cheese from small and medium-sized producers.

Hypothesis 2: There exist differences in preferences between unsmoked steamed cheese from small and medium-sized producers.

MATERIAL AND METHODOLOGY

Samples

8 samples of unsmoked steamed cheese (nite and parenica) were obtained directly from medium-sized producers and small farms on the day of production. Samples A, B, C, and E (4 samples) were produced by medium-sized producers, and samples D, F, G, and H (4 samples) were manufactured in small farms. All of the samples of unsmoked steamed cheese can be seen in Figure 1.

Sensory analysis

The sensory evaluation was performed in a sensory laboratory designed according to **ISO 8589:2007** located in the Slovak University of Agriculture in Nitra. Samples were coded with 3-digit number codes and served on white ceramic plates at temperature 20 ±2 °C. Samples for evaluation were prepared by cutting the cheese into smaller portions. Samples were evaluated the day after production. 10 assessors who were all students and employees of the Slovak University of Agriculture in Nitra took part in this evaluation. All of them had previous experiences with a sensory evaluation of dairy products.

A nine-point hedonic scale was used for sensory evaluation, where 1 was very bad and 9 was very good. Evaluated attributes were color, odor, texture, flavor, and overall appearance. Wiliams Latin square design was used for sample randomization **(Williams, 1949; Wang, Wang, and Gong, 2009)**. Mineral water was used as a palate cleanser to neutralize the taste between samples.

Preference mapping

Internal mapping data were obtained from the sensory analysis of samples. Data needed for external mapping were collected from an online questionnaire with 120 responses. Respondents were asked to score the samples according to their personal preferences on a nine-point scale.

Statistical analysis

For analysis of sensory data was used Shapiro-Wilk normality test and parametric t-test (RStudio software, version 1.3.1093, R Foundation for Statistical Computing, Vienna, Austria). Data analysis for PCA and Preference mapping was carried out with XLSTAT statistical software (version 2020.5.1 by Addinsoft). Internal data from the sensory evaluation were processed with PCA (Principal Component Analysis) and external data from the online questionnaire were used for AHC (Agglomerative Hierarchical Clustering).

RESULTS AND DISCUSSION

Slovenská parenica, Oravský korbáčik, Zázrivský korbáčik, and Zázrivské vojky granted the PGI designation (Protected Geographical Indication) in 2008 – 2014. Regarding the PDO, PGI, and TSG labels there have been reported numerous problems. Food adulteration has many forms e.g. substitution, mislabelling, masking of origin, decreasing quality of food products, intentional misinterpretation, artificial enhancement, etc. **(Fikselová et al., 2020)**. According to **Singh and Gandhi (2015)**, milk and milk products are typically adulterated with the use of preservatives and adulterants (benzoic acid, water addition, hydrogen peroxide, salicylic acid, etc.).

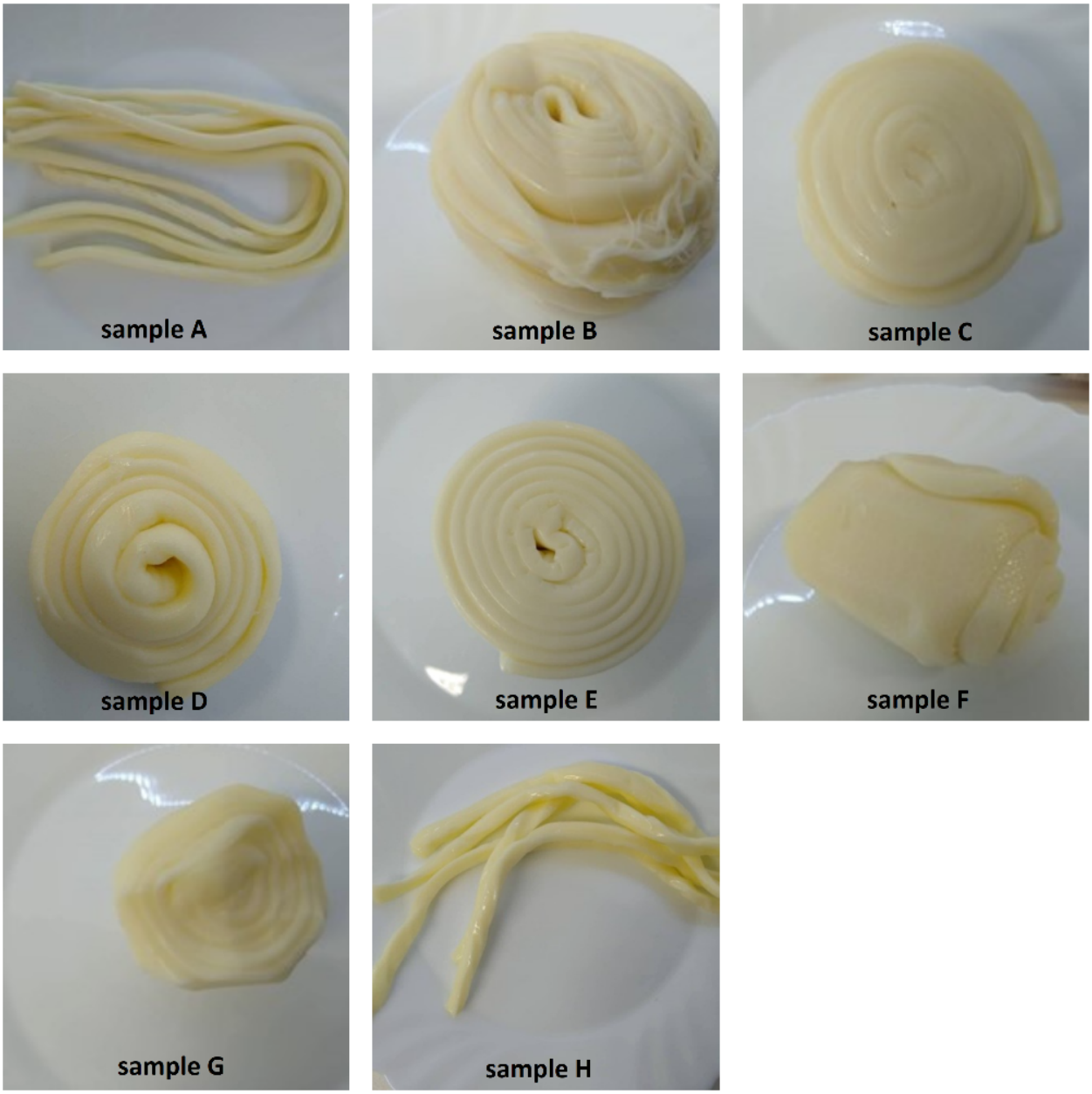

Figure 1 Samples (A – H) of unsmoked steamed cheeses.

Table 1 Summary table of sensory analysis.

	Colour		Odour		Texture		Flavour		Overal appearance	
Sample	**Average**	**Variance**	**Average**	**Variance**	**Average**	**Variance**	**Average**	**Variance**	**Average**	**Variance**
A	7.00	2.00	7.00	2.96	7.00	3.16	7.00	3.49	7.00	2.01
B	6.00	2.81	6.00	4.00	8.00	2.00	6.00	2.44	7.00	1.96
C	6.00	3.29	7.00	2.00	7.00	4.00	6.00	3.25	7.00	2.24
D	6.00	3.58	7.00	2.00	7.00	2.00	6.00	5.01	7.00	4.44
E	7.00	1.96	6.00	4.00	7.00	4.00	6.00	4.01	7.00	1.69
F	6.00	2.49	5.00	4.00	7.00	1.00	4.00	2.89	5.00	3.80
G	5.00	5.44	4.00	2.00	7.00	1.00	3.00	3.21	4.00	4.84
H	6.00	3.85	5.00	6.00	6.00	5.00	6.00	3.76	6.00	2.81
p-value	0.1857		0.00111		0.04885		0.01456		0.02449	

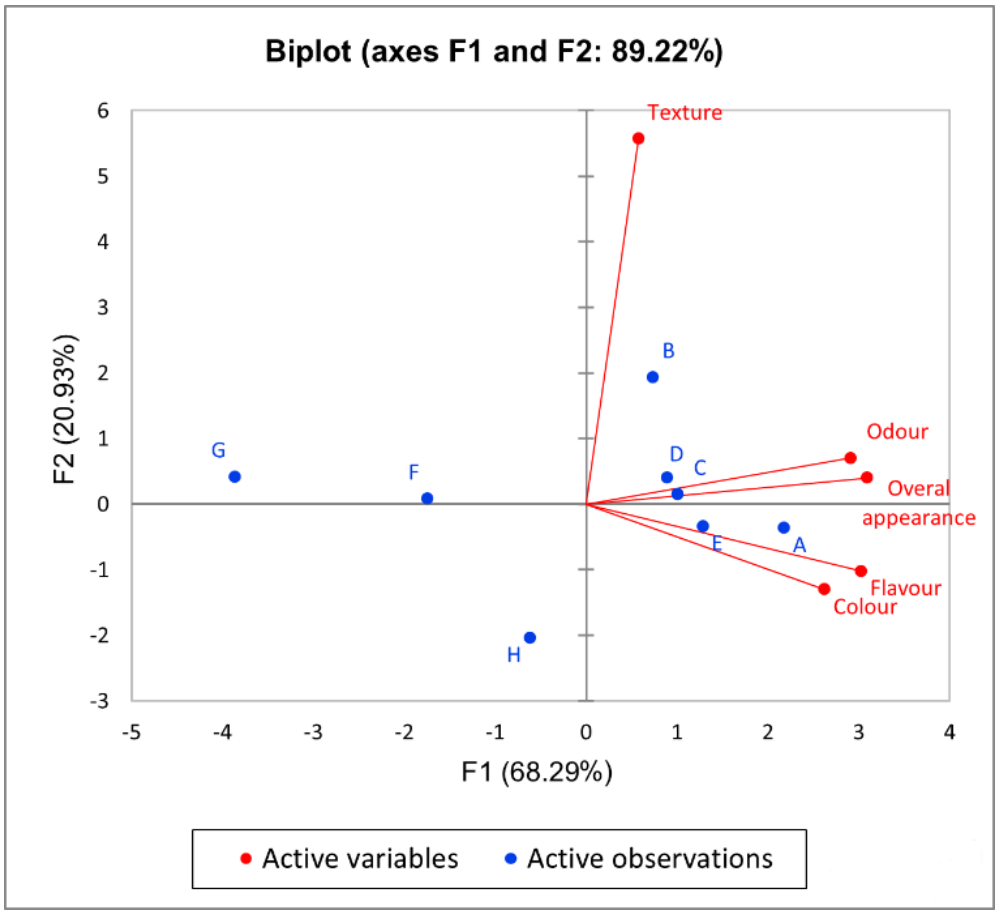

Figure 2 PCA map of samples.

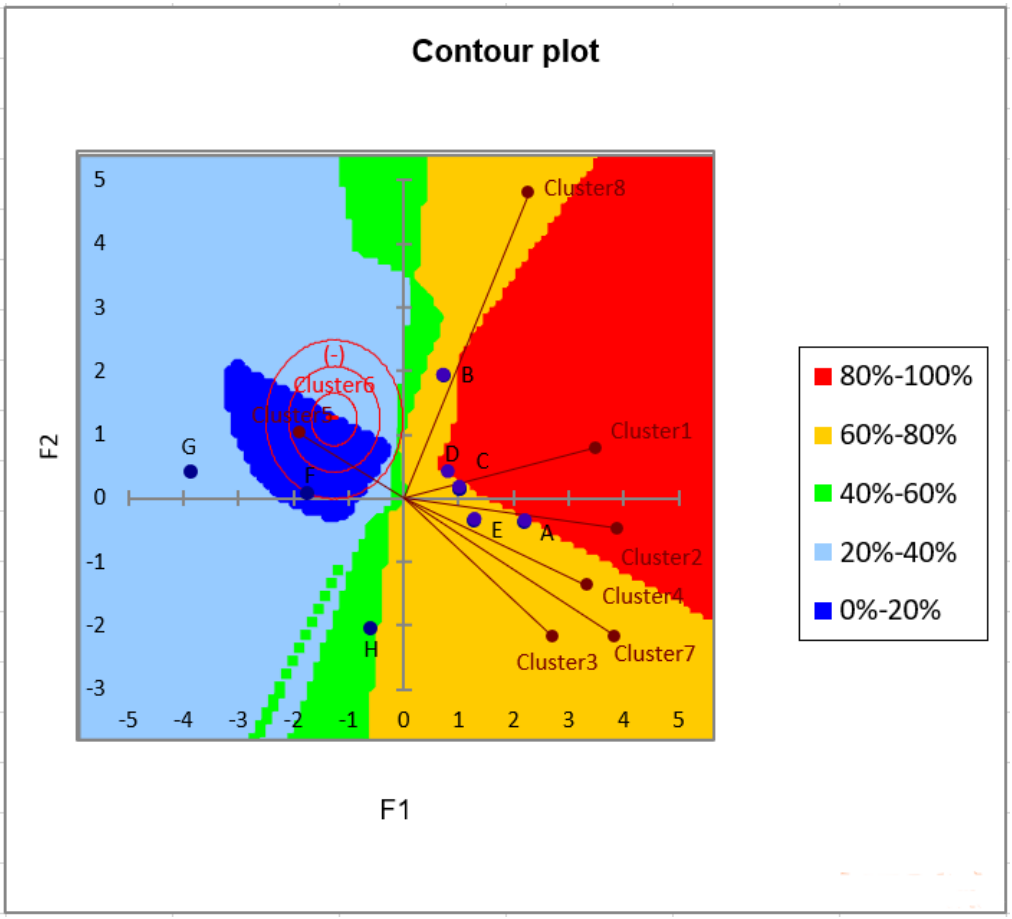

Figure 3 Visualization of the consumers preferences with preference map.

Characteristic steamed cheese production and starter cultures are mentioned in the work of **Onipchenko et al. (2012)**, **Štefániková et al. (2019),** and **Zimanová et al. (2016)**.

The summary of sensory analysis results is presented in Table 1. There was no significant difference between samples from medium-sized and small farms in the parameter color (p >0.05). Statistically significant differences were detected in the attributes odor, texture, flavor, and overall appearance (p <0.05), where samples with higher consumer acceptance were evaluated samples from medium-sized farms in all parameters (Table 1). Authors **Semjon et al. (2019)** detected differences between experimental parenica cheese samples in every evaluated sensory parameter in the early stage of storage. They also observed statistically significant differences (p <0.05) between samples in sensory parameters after storage. Similar sensory attributes and a nine-point scale were used by authors **Tauferova et al. (2014)** in their study of the sensory texture of ketchup.

PCA map of evaluated unsmoked steamed cheese samples can be seen in Figure 2. Samples were divided into two groups, the first group consisted of samples from medium-sized farms (A, B, C, E) and one sample from the small farm (D), the second group consisted of three samples of cheese from small farms (F, G, H). All of the evaluated parameters were characteristic for the first group of samples. Sample B had the highest rating in texture parameters. Samples A, C, D, and E obtained high values in the evaluation of odor, overall appearance, flavor, and color. On the other hand, there were samples from the second group (F, G, H) where sample H had the worst texture, it was very solid and firm. Samples in the second group also lacked odor, flavor, color, and overall appearance attributes.

The preference map of evaluated unsmoked cheese can be seen in Figure 3. In the highest consumer preference zone (80 – 100%) were partly located samples A, C, and D, these samples were also located in the second preference zone of 60 – 80%. These samples were preferred the most by the respondents. Samples B and E were located in the zone of preference 80 – 60% and sample H can be seen in the green field with preference 40 – 60%. As less preferred sample consumers chose sample G (20 – 40%) and the least preferred sample was sample F (0 – 20%). The preference map corresponded with the results of sensory analysis and samples obtained from medium-sized farms had higher preferences from consumers than samples coming from small farms. Preference mapping was previously used in the analysis of a wide variety of foodstuffs e.g. ham **(Benešová et al., 2019)**, parenica steamed cheese **(Semjon et al., 2019)**, raw garlic **(Drdolová, Martišová and Benešová, 2019)**, fresh fruit **(Villamor et al., 2013; Jaeger et al., 1998; Lado et al., 2010)**, vegetable **(Sinesio et al., 2010)**, etc.

In a similar study authors **Drdolová, Martišová and Benešová (2019)** evaluated 10 varieties of winter garlic using PCA and preference mapping techniques. According to their findings, assessors tend to overestimate samples with attractive appearance and to focus on textural parameters of samples.

According to **Zajác et al. (2019)** quality of a traditional product can vary from farm to farm. It is mainly caused by differences in cheese-making technology e.g. temperature of milk pasteurization, type, and a dose of rennet, amount of salt, smoking, drying process, etc. In their work, **Zajác et al. (2019)** studied differences in textural and sensory characteristics of typical Slovak cheese – oštiepok. They confirmed significant differences (p <0.05) between the samples from different regions in both textural and sensory attributes.

The different temperature used during cheese maturation affects the content of the microorganisms **(Kunová et al., 2015)**. Microbial count increases when using insufficient pasteurization and there is a possibility of cross-contamination **(Zajác et al., 2019)**.

Authors **Ducková et al. (2019)** in their work studied the effect of somatic cell count (SCC) in milk intended for parenica cheese making. They compared the number of somatic cells in milk from small dairy farms with milk from industrial companies and they did not find the statistically significant difference (p >0.05). However lower SCC was observed in samples from industrial dairies. According to **Hachana, Znaidi and M´Hamdi (2018)** higher values of SCC can have a negative effect on dairy products shelf life and sensory characteristics.

CONCLUSION

In our study, we proved the existence of differences in sensory attributes between samples manufactured in small dairy farms and samples from medium-sized industry farms. The differences at the statistically significant level (p <0.05) were detected in the parameters odor, texture, flavor, and overall appearance. From the map of principal component analysis can be seen that samples from industry farms obtained high values in the evaluation of all sensory attributes and samples from small dairy farms (except sample D) lacked in these parameters. The results obtained from preference mapping also coincided with the above mention analyses and therefore the most preferred samples came from medium-sized industry farms.

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origin and protected geographical indications (Chamomilla Bohemica (PDO), Vlaams-Brabantse tafeldruif (PDO), Slovenská parenica (PGI), Cipollotto Nocerino (PDO)).

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